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[Continued on next page]

(54) Title: ANTIBODIES AND ANTIBODY-DRUG CONJUGATES

(57) Abstract: This application provides antibodies and antigen binding fragments thereof which are capable of specifically binding the 5T4 cell surface antigen, antibody-drug conjugates, and antibody-imaging agent conjugates, as well as means and methods for producing and using them.

FIG. 1

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5T4-A07_GL
EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAWMS----WVRQAPGKGLEWIGRIRSKA---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0060
EVQLVESGGGLVKPGGSLRLSCAASGEWASSQWMS----WVRQAPGKGLEWIGRIRSKA---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0065
EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAWMS----WVRQAPGKGLEWIGRIRAQR---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0068
EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAWMS----WVRQAPGKGLEWIGRIRSKA---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0069
EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAWMS----WVRQAPGKGLEWIGRIRSKA---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0107
EVQLVESGGGLVKPGGSLRLSCAASGEWASSQWMS----WVRQAPGKGLEWIGRIRSKA---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0108
EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAWMS----WVRQAPGKGLEWIGRIRAQR---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0112
EVQLVESGGGLVKPGGSLRLSCAASGFTFSNAWMS----WVRQAPGKGLEWIGRIRASQ---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS

5t4_0113
EVQLVESGGGLVKPGGSLRLSCAASGFTFQTYPMH----WVRQAPGKGLEWIGRIRSKA---DGGTTDSAAPVKG
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTVDRRNYYGM--------------DVWGQGTTVTVSS
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DESCRIPTION

Field

[001] This application relates to the field of biotechnology and medicine.

Background

Antibody Drug Conjugates

[002] Antibody-drug conjugates (ADCs) represent a promising new therapeutic approach to more effectively treat cancer while reducing drug-related toxicities by combining the specificity of an antibody with the potency of cytotoxic small molecules. An ADC consists of a warhead that may be a small molecule that has been chemically modified to contain a linker that is used to conjugate the drug to the antibody. Cytotoxicity is induced when the ADC binds to the antigen surface of a target-positive cancer cell, is internalized and trafficked to the lysosome where the warhead is released following either proteolysis of a cleavable linker (for example by cathepsin B found in the lysosome) or through proteolytic degradation of the antibody when a non-cleavable linker is used to attach the warhead to the antibody. The warhead then translocates out of the lysosome and into the cytosol where it can then bind to its target, depending on its mechanism of action. Typically these warheads induce cell cycle arrest which subsequently leads to apoptosis. Corresponding conjugates containing imaging agents also represent a promising new way to detect cancer cells *in vivo* or *in vitro.*

Trophoblast glycoprotein – 5T4

[003] This application considers ADC and antibody-imaging agent conjugates targeting 5T4. 5T4 (also known as trophoblast glycoprotein) is a 72 kDa glycoprotein that is an oncofetal antigen and as such, it is typically only expressed during embryonic development, whereas expression in normal adult tissues is very limited (*Hole and Stern*, Br J Cancer 57:239-246 (1988)). Expression of 5T4, however, is significantly upregulated in many types of carcinomas including, but not limited to, breast cancer, gastric cancer, prostate cancer, colorectal carcinoma,

ovarian cancer and lung cancer and its expression has been correlated with poor prognosis in multiple indications. *Al-Taei et al.*, Lung Cancer 77(2):312-8 (2012); *Griffiths et al.*, Br J Cancer 93(6):670-7 (2005); *Mulder et al.,* Clin Cancer Res 3(11):1923-30 (1997); *Southall et al.*, Br J Cancer 61(1):89-95 (1990); *Starzynska et al.*, Br J Cancer 66(5):867-9 (1992); *Starzynska et al.*, Br J Cancer 69(5):899-902 (1994); *Wrigley et al.*, Int J Gyncol Cancer 5(4):269-274 (1995)..

[004] The biology of 5T4 in normal and pathologic tissues is not well understood. The extracellular domain of 5T4 contains several leucine-rich repeats which have been implicated with protein-protein interactions which can promote cell-cell interactions. *Myers et al.*, J Biol Chem 269(12)9319-24 (1994). However no ligand or co-receptor has been identified for 5T4 to date, though 5T4 appears to regulate CXCR4-mediated chemotaxis and may be involved in regulating Wnt signaling in embryonic development. *Kagermeier-Schenk et al.*, Dev Cell 21(6):1129-43 (2011); *Southgate et al.*, PLoS One 5(4):e9982 (2010). 5T4 has a short cytoplasmic domain consisting of 44 amino acids and aside from a serine-asparagine-valine motif that is implicated in binding PDZ-containing molecules; there are no classical signal transduction motifs contained in the sequence. *Awan et al.*, Biochem Biophys Res Commun 290(3):1030-6 (2002); *Myers* (1994). Studies with overexpression or knockdown of 5T4 have demonstrated that it likely plays a role in adhesion, cytoskeletal organization and motility and it is implicated in the epithelial-to-mesenchymal transition (EMT) that is associated with cancer progression to a metastatic phenotype. *Awan* (2002); *Carsberg et al.*, J Cell Sci 108(pt. 8):2905-16 (1995); *Carsberg et al.*, Int J Cancer 68(1):84-92 (1996); *Spencer et al.*, Mol Biol Cell 18(8):2838-51 (2007). 5T4 knockout mice are viable, though adults have a number of structural defects in the brain as a result of changes that occur during brain development in utero. *Southgate* (2010).

[005] 5T4 is also expressed on cancer stem cells (CSCs) which are hypothesized to be responsible for chemotherapeutic resistance and recurrence of cancer. Therefore targeting 5T4-positive CSCs could eliminate this population of the tumor and prevent recurrence resulting in more durable responses. Taken together, an ADC targeting 5T4, which is expressed in a wide variety of various carcinomas, could potentially represent a broadly-applicable, and effective therapeutic.

[006] Antibodies to 5T4 are thus promising targets for targeting drugs to cancer cells expressing this marker. Antibodies to 5T4 would also provide benefits in detecting cells expressing this receptor, whether *in vivo* or *in vitro*. Therefore, there is a need in the art for alternative antibodies and therapies against 5T4. There is also a need for antibody-drug conjugates and antibody-imaging agent conjugates.

[007] Several anti-5T4 antibodies have been described, including mAb5T4, also called the H8 antibody, which recognizes a conformational epitope of the 5T4 antigen (Shaw et al. (2002) Biochem. J. 363: 137-45, PCT International Publication No. WO98/55607), a rat monoclonal antibody (Woods et al. (2002) Biochem. J. 366: 353-65), and a mouse monoclonal antibody called 5T4 (U.S. Patent No. 5,869,053). Single chain anti-5T4 antibodies have also been described, as well as fusion proteins that include anti-5T4 antibody sequences fused to a therapeutic molecule. For example, anti-5T4 antibody sequences fused to the human IgGI constant domain or to the extracellular domain of murine B7.1 induces cytolysis of 5T4-expressing tumor cell lines (Myers et al. (2002) Cancer Gene Ther. 9: 884-896, Shaw et al. (2000) Biochim. Biophys. Acta. 1524: 238-246; U.S. Patent Application Publication No. 2003/0018004). Similarly, a single chain anti-5T4 antibody fused to a superantigen may stimulate T cell-dependent cytolysis of non-small cell lung carcinoma cells in vitro (Forsberg et

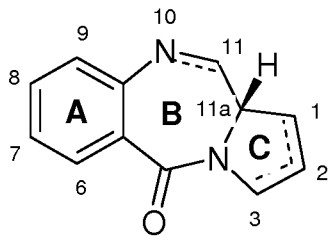
al. (2001) Br. J. Cancer 85: 129-136). A phase I clinical trial using PNU-214936, a murine Fab fragment of the monoclonal antibody 5T4 fused to a mutated superantigen staphylococcal enterocytotoxin A (SEA), showed limited toxicity and some anti-tumor response (Cheng et al. (2004) J. Clin. Oncol. 22(4):602-9). As an alternate therapeutic approach, recombinant 5T4 vaccines are also suggested for the treatment of cancers (Mulryan et al. (2002) MoI. Cancer Ther. 1 : 1 129-37; UK Patent Application Publication Nos. 2,370,571 and 2,378,704; EP Patent Application Publication Nos. EP 1 ,160,323 and 1 ,152,060). More recently WO 2007/106744 has contemplated the use of an anti-5T4 antibody as a part of an antibody drug conjugate.

Cytotoxic Agents

[008] A number of classes of cytotoxic agents are known to have potential utility as warheads in ADC molecules. These include but are not limited to amanitins, auristatins, daunomycins, doxorubicins, duocarmycins, dolastatins, enediynes, lexitropsins, taxanes, puromycins, maytansinoids, vinca alkaloids, tubulysins and pyrrolobenzodiazepines. Examples of such cytotoxic agents are AFP, MMAF, MMAE, AEB, AEVB, auristatin E, paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretatstatin, chalicheamicin, maytansine, DM-1, vinblastine, methotrexate, and netropsin.

[009] Some pyrrolobenzodiazepines (PBDs) have the ability to recognise and bond to specific sequences of DNA; the preferred sequence is PuGPu. The first PBD antitumour antibiotic, anthramycin, was discovered in 1965 (Leimgruber, et al., J. Am. Chem. Soc., 87, 5793-5795 (1965); Leimgruber, et al., J. Am. Chem. Soc., 87, 5791-5793 (1965)). Since then, a number of naturally occurring PBDs have been reported, and over 10 synthetic routes have been developed to a variety of analogues (Thurston, et al., Chem. Rev. 1994, 433-465 (1994); Antonow, D. and Thurston, D.E., Chem. Rev. 2011 111 (4), 2815-2864). Family members

include abbeymycin (Hochlowski, et al., J. Antibiotics, 40, 145-148 (1987)), chicamycin (Konishi, et al., J. Antibiotics, 37, 200-206 (1984)), DC-81 (Japanese Patent 58-180 487; Thurston, et al., Chem. Brit., 26, 767-772 (1990); Bose, et al., Tetrahedron, 48, 751-758 (1992)), mazethramycin (Kuminoto, et al., J. Antibiotics, 33, 665-667 (1980)), neothramycins A and B (Takeuchi, et al., J. Antibiotics, 29, 93-96 (1976)), porothramycin (Tsunakawa, et al., J. Antibiotics, 41, 1366-1373 (1988)), prothracarcin (Shimizu, et al, J. Antibiotics, 29, 2492-2503 (1982); Langley and Thurston, J. Org. Chem., 52, 91-97 (1987)), sibanomicin (DC-102)(Hara, et al., J. Antibiotics, 41, 702-704 (1988); Itoh, et al., J. Antibiotics, 41, 1281-1284 (1988)), sibiromycin (Leber, et al., J. Am. Chem. Soc., 110, 2992-2993 (1988)) and tomamycin (Arima, et al., J. Antibiotics, 25, 437-444 (1972)). PBDs are of the general structure:



[010] They differ in the number, type and position of substituents, in both their aromatic A rings and pyrrolo C rings, and in the degree of saturation of the C ring. In the B-ring there is either an imine (N=C), a carbinolamine(NH-CH(OH)), or a carbinolamine methyl ether (NH-CH(OMe)) at the N10-C11 position which is the electrophilic centre responsible for alkylating DNA. All of the known natural products have an (S)-configuration at the chiral C11a position which provides them with a right-handed twist when viewed from the C ring towards the A ring. This gives them the appropriate three-dimensional shape for isohelicity with the minor groove of B-form DNA, leading to a snug fit at the binding site (Kohn, In Antibiotics III. Springer-Verlag, New York, pp. 3-11 (1975); Hurley and Needham-VanDevanter, Acc. Chem. Res., 19, 230-237

(1986)). Their ability to form an adduct in the minor groove, enables them to interfere with DNA processing, hence their use as antitumour agents.

Objects of the Present Invention

[011] It is an object of the present invention to provide means and methods for binding to 5T4 cell surface receptors, treating cancer or inhibiting cancer cell growth using an antibody-drug conjugate, and detecting cells that exhibit the 5T4 cell surface receptor with an antibody-imaging agent conjugate. The detection method can be used in vitro or in vivo.

[012] As discussed herein, the 5T4-ADCs are composed of a human monoclonal antibody (mAb) that recognizes 5T4 conjugated to a tubulysin or pyrrolobenzodiazepine dimer (PBD) warhead. Upon binding to 5T4 on the surface of cancer cells, 5T4-ADC is internalized and trafficked to the lysosome, where proteolytic degradation of a cleavable dipeptide linker releases the tubulysin or PBD dimer warhead. The tubulysin warhead inhibits tubulin polymerization and subsequently triggers the apoptotic process. The PBD warhead translocates to the nucleus where it crosslinks DNA, preventing replication during mitosis, damaging DNA by inducing single strand breaks and subsequently leading to apoptosis. 5T4-ADCs disclosed herein have very potent activity against tumor cells in a target-dependent manner in vitro, and are able to induce regression of 5T4-positive tumors in vivo. Given the broad expression of 5T4 in carcinomas and the correlation between expression and poor prognosis, the 5T4-ADCs could have potential utility in a number of oncology indications. Al-Taei (2012); Griffiths (2005); Mulder (1997); Southall (1990); Starzynska (1992); Starzynska (1994); Wrigley (1995).

SUMMARY OF THE INVENTION

[013] In accordance with the description, the present disclosure provides an antibody, or an antigen binding fragment thereof, capable of specifically binding 5T4 cell surface antigen comprising:

a. a heavy chain variable region CDR1 sequence comprising a sequence $GX_1X_2X_3X_4X_5X_6DX_7X_8X_9$, wherein
X_1=F, D or E,
X_2=T, W or G,
X_3=F, A S or T,
X_4= S, A, Q, H, T, G or K,
X_5=N, T, Q, R, A, L, P or S,
X_6=Q, Y, A, L, F, M or S,
X_7=P, W, Q, or L,
X_8=M, L, I, G or V,
X_9=H, S or T,

b. a heavy chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6DX_7X_8X_9X_{10}X_{11}$SAAPVKG, wherein
X_1=R, A, or Q,
X_2=I, V, L, M, or Q,
X_3=R or K,
X_4=A, S, or G,
X_5=Q, K, E, S, V, I, H, L, R, or Y,
X_6=A, Q, R, L, M,
X_7=G, N, E, L,
X_8=G, A, D, E, Q,
X_9=T, E, Q, R, V, W,
X_{10}=T, Q, V, I, P, and
X_{11}=D, Q, V, F, W;

c. a heavy chain variable region CDR3 sequence comprises a sequence that differs by no more than one or two amino acid from the sequence VDRRNYYGMDV (SEQ ID NO: 5;

d. a light chain variable region CDR1 sequence comprises a sequence that differs by no more than one or two amino acids from the sequence RASQGIRNDLG (SEQ ID NO: 12);

e. a light chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6$S, wherein
X_1=A or N,
X_2=A, G, S, T, or V,
X_3=S, W, D, G, N, Q, H, or T,

X_4=R, Q, T, S, H, or K,
X_5=R, L, P, W, H, K, M, N, Q, S, T, or V, and
X_6=D, M, S, or V; and

f. a light chain variable region CDR3 sequence comprising a sequence $X_1QX_2X_3X_4X_5PWX_6$, wherein
X_1=L or V,
X_2=Q, M, or L,
X_3=R, K, Y, F, N, A, H, T, or W,
X_4=R, K, A, N, Q, L, M, S, or T,
X_5=M, T, V, A, I, Q, Y, L, P, K, R, or S, and
X_6=T, R, M, V, A, K, or S.

[014] In some aspects the antibody, or an antigen binding fragment thereof, capable of specifically binding 5T4 cell surface antigen comproises one or more CDR sequences independently selected from:

heavy chain variable region CDR1 sequences comprising NAWMS (SEQ ID NO: 3), SQWMS (SEQ ID NO: 39), or TYPMH (SEQ ID NO: 129).

heavy chain variable region CDR2 sequences comprising RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4) or RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58).

a heavy chain variable region CDR3 sequence comprising VDRRNYYGMDV (SEQ ID NO: 5).

a light chain variable region CDR1 sequence comprising RASQGIRNDLG (SEQ ID NO: 12),

light chain variable region CDR2 sequences comprising AASSLQS (SEQ ID NO: 13) or AGWQRDS (SEQ ID NO: 85);

light chain variable region CDR3 sequences comprising LQQNSYPWT (SEQ ID NO: 14), LQQRRMPWT (SEQ ID NO: 122), LQQYRVPWT (SEQ ID NO: 50), or LQMRRTPWT (SEQ ID NO: 104).

[015] In some aspects, the IgG Fc domain, for example an IgG1 Fc domain, is a native (wild type) domain. In other aspects, the Fc domain is a mutant IgG domain, for example, a mutant IgG1, IgG2, IgG3, or IgG4 domain. In some specific aspects, the mutant Fc domain is a mutant IgG1 Fc domain. The mutant FC region may have been modified to reduce, ADCC activity, improve half life and/or provide a derivitizable functional group. For instance, a

derivatizable functional group may be the sulfhydryl side chain of a cysteine amino acid introduced at one or more of the following positions 239, 248, 254, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 440, 441, 442, 443 and 446, wherein the numbering corresponds to the EU index in Kabat.

[016] In an aspect an antibody-drug conjugate is provided wherein the antibody-drug conjugate comprises:

(i) an antibody, or antigen binding fragment thereof, capable of specifically binding 5T4 cell surface antigen;

(ii) a chemotherapeutic agent; and

(iii) optionally a linker.

[017] In some aspects the chemotherapeutic agent is selected from the group consisting of amanitin, auristatin, daunomycin, doxorubicin, duocarmycin, dolastatin, enediyne, lexitropsin, taxane, puromycin, maytansinoid, vinca alkaloid, tubulysin , pyrrolobenzodiazepine, AFP, MMAF, MMAE, AEB, AEVB, auristatin E, paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretatstatin, chalicheamicin, maytansine, DM-1, vinblastine, methotrexate, and netropsin. In some aspects the chemotheraputtic agent is a tubulysin, e.g. tubulysin 1508, or a pyrrolobenzodiazepine, e.g. Comp64 (Compound 64 – see Example16) or Comp96 (Compound 96 – see Example 20).

[018] In an aspect an antibody-drug conjugate is provided wherein the antibody-imaging conjugate comprises:

(i) an antibody, or antigen binding fragment thereof, capable of specifically binding 5T4 cell surface antigen;

(ii) an imaging agent; and

(iii) optionally a linker.

[019] In aspect, nucleic acids, vectors, host cells and host cell lines capable of expressing antibodies, or antigen binding fragments thereof, capable of specifically binding 5T4 cell surface antigen are provided.

[020] In an aspect a method of inhibiting growth of a cancer cell comprising exposing a cancer to an anti-5t4 antibody-drug conjugate is provided. In an aspect a method of treating cancer in a subject comprising administering an anti-5t4 antibody-drug conjugate is provided.

[021] In an aspect a method of detecting a cancer cell expressing the 5T4 cell surface antigen comprising exposing a cell toan anti-5t4 antibody-imaging agent conjugate is provided.

[022] Additional objects and advantages will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice. The objects and advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[023] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the claims.

[024] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one (several) embodiment(s) and together with the description, serve to explain the principles described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[025] Figure 1 provides a heavy chain sequence alignment. CDRs are shown with underlining and mutations are shown with gray shading.

[026] Figure 2 provides a light chain sequence alignment. CDRs are shown with underlining and mutations are shown with gray shading.

[027] Figure 3 shows binding of ZY07N8-A07 (5T4A07) as a Phynexus purified chimeric antibody (murine Fc) to Eu-TBP-K human and cyno 5T4 ECD Fc using HTRF.

[028] Figure 4 shows an example of binding of ZY07N8-A07(5T4A07) purified chimeric mab with murine Fc to Eu-TBP-K 5T4 ECD FC using HTRF.

[029] Figure 5 shows binding of ZY07N8-A07(5T4A07) as a purified human mab to Eu-TBP-K human 5T4 ECD Fc using HTRF.

[030] Figure 6 shows specificity ELISA ZY07N8-A07(5T4A07) demonstrating specific binding to human 5T4.

[031] Figure 7 provides an example of inhibition of biotinylated ZY07N8-A07(5T4A07) IgG binding to EU-TBP-K human 5T4 ECD Fc by hisprep scFv using HTRF.

[032] Figure 8 provides an example of inhibition of biotinylated ZY07N8-A07(5T4A07) IgG binding to EU-TBP-K cyno 5T4 ECD Fc by hisprep scFv using HTRF.

[033] Figure 9 provides an example of inhibition of biotinylated ZY07N8-A07(5T4A07) IgG binding to Eu-TBP-K human 5T4 ECD Fc by human IgGs using HTRF.

[034] Figure 10 provides an example of inhibition of biotinylated ZY07N8-A07(5T4A07) IgG binding to EU-TBP-K cyno 5T4 ECD Fc by human IgGs using HTRF

[035] Figure 11 shows a specificity ELISA 5T4_0108 demonstrating specific binding to human 5T4.

[036] Figures 12A-I shows Cell-Surface binding of parental anti-5T4 antibodies to various human cancer cell lines

[037] Figures 13A-J show cell-surface binding of parental anti-5T4 antibodies to cell lines expressing human or cyno 5T4.

[038] Figure 14A shows internalization of parental anti-5T4 antibodies by DU 145 human prostate cancer cells. Figure 14B parental anti-5T4 antibodies tested in the Hum-Zap saporin-conjugate assay induce cytotoxicity of 5T4-positive DU 145 prostate cancer cells

[039] Figure 15 shows that internalization rates of parental A07, affinity-optimized 108(FC) antibodies and a tubulysin conjugate of 108(FC) are comparable.

[040] Figure 16 illustrates lysosomal localization of 108(FC) following internalization by DU 145-LAMP-1 prostate cancer cells.

[041] Figures 17A and B demonstrates that affinity-optimized ADCs show significantly improved cytotoxicity over parental antibodies against DU 145 human prostate cancer cells.

[042] Figures 18A-B shows cytotoxicity of ADCs bearing 108(FC) (representative assay).

[043] Figure 19A shows that 108(FC)-PBD and 108(FC)-1508 conjugates have similar cytotoxicity against DU 145 human prostate cancer cells *in vitro*. Figure 19B shows that 108(FC)-Comp64 and 108(FC)-Comp96 had comparable activity against MDA-MB-361 cells and each was more potent than 108(FC)-1508.

[044] Figure 20 shows 108(239iCys) conjugated to tubulysin in a site-specific manner potently inhibits the growth of MDA-MB-361 cells *in vitro*.

[045] Figure 21 shows that 108(FC)-Comp64 induces bystander kill activity of 5T4-negative tumor cells.

[046] Figure 22 shows that 108(FC)-Comp96 induces bystander kill activity of 5T4-negative tumor cells.

[047] Figure 23 shows that 108(FC)-Comp64 produces more durable regressions of DU 145 xenografts than 108(FC)-1508.

[048] Figure 24 shows dose-dependent regression of DU 145 prostate xenografts with treatment with 108(FC)-PBD conjugates.

[049] Figure 25A shows dose-dependent regression of NCI-N87 gastric xenografts upon single administration of 108(FC)-Comp64. Figure 25B shows dose-dependent regression of NCI-N87 gastric xenografts with treatment with 108(FC)-Comp64. Figure 25C shows dose-dependent regression of MDA-MB-361 breast cancer xenografts upon single administration with 108(FC)-Comp64. Figure 25D shows dose-dependent regression of MDA-MB-361 breast cancer xenografts upon treatment with 108(FC)-Comp64.

[050] Figure 26A shows dose-dependent regression of H1975 lung cancer xenografts upon single administration of 108(FC)-Comp64. Figure 26B shows dose-dependent regression of H1975 lung cancer xenografts upon treatment with 108(FC)-Comp64.

[051] Figure 27A shows comparable Efficacy with Single vs. Fractionated Doses of 108(FC)-Comp64 in Gastric PDX Model with High/Heterogeneous 5T4 Expression. Figure 27B shows comparable Efficacy with Single vs. Fractionated Doses of 108(FC)-Comp64 in Gastric PDX Model with Low/Homogenous 5T4 Expression.

[052] Figure 28 shows that the PBD warhead of Comp64 potently targets CSCs *in vitro.*

[053] Figure 29 illustrates that 108(FC)-Comp64 potently inhibits the growth of DU 145 CSCs *in vitro.*

[054] Figure 30 illustrates that 108(FC)-Comp64 potently inhibits the growth of Sum159 CSCs *in vitro*.

[055] Figure 31 demonstrates that a single dose of 5T4-108, unlike the Auristatin armed A1 mAb ADC, reduces the *in vivo* CSC population.

[056] Figures 32A-B illustrate isolectric point determination for 5T4-108-239iCys and 5T4-108-239iCys-1508.

[057] Figures 33A-B illustrate differential scanning calorimetry analysis.

[058] Figure 34 shows size exclusion chromatography analysis for 5T4-108-239iCys and 5T4-108-239iCys-1508.

[059] Figure 35 shows hydrophobic Interaction Chromatography for 5T4-108-239iCys and 5T4-108-239iCys-1508.

[060] Figure 36 shows reduced glycosylated reverse phase analysis of 5T4-108-239iCys and 5T4-108-239iCys-1508.

[061] Figure 37 shows non-GLP tox buffer stability assessment including 3X F/T.

[062] Figure 38 shows mouse serum stability of 5T4-108-239iCys-1508

DESCRIPTION OF THE SEQUENCES

[063] Table 1 provides a listing of certain sequences referenced in present embodiments. While each antibody is assigned sequence numbers for each CDR for an abundance of clarity, some of the CDRs are the same for multiple antibodies. The remainder of the specification and claims will reference the first instance of that CDR in the sequence listing.

Table 1: Description of the Sequences

SEQ ID NO.	Source or Sequence	Description
1	ZY07N8-A07 (also referred to herein as 5T4A07)	VH DNA
2	ZY07N8-A07	VH PRT
3	ZY07N8-A07	HC CDR1 PRT
4	ZY07N8-A07	HC CDR2 PRT
5	ZY07N8-A07	HC CDR3 PRT
6	ZY07N8-A07	HC FW1 PRT
7	ZY07N8-A07	HC FW2 PRT
8	ZY07N8-A07	HC FW3 PRT
9	ZY07N8-A07	HC FW4 PRT
10	ZY07N8-A07	VL DNA
11	ZY07N8-A07	VL PRT
12	ZY07N8-A07	LC CDR1 PRT
13	ZY07N8-A07	LC CDR2 PRT
14	ZY07N8-A07	LC CDR3 PRT

15	ZY07N8-A07	LC FW1 PRT
16	ZY07N8-A07	LC FW2 PRT
17	ZY07N8-A07	LC FW3 PRT
18	ZY07N8-A07	LC FW4 PRT
19	ZY07N8-A07_GL	VH DNA
20	ZY07N8-A07_GL	VH PRT
21	ZY07N8-A07_GL	HC CDR1 PRT
22	ZY07N8-A07_GL	HC CDR2 PRT
23	ZY07N8-A07_GL	HC CDR3 PRT
24	ZY07N8-A07_GL	HC FW1 PRT
25	ZY07N8-A07_GL	HC FW2 PRT
26	ZY07N8-A07_GL	HC FW3 PRT
27	ZY07N8-A07_GL	HC FW4 PRT
28	ZY07N8-A07_GL	VL DNA
29	ZY07N8-A07_GL	VL PRT
30	ZY07N8-A07_GL	LC CDR1 PRT
31	ZY07N8-A07_GL	LC CDR2 PRT
32	ZY07N8-A07_GL	LC CDR3 PRT
33	ZY07N8-A07_GL	LC FW1 PRT
34	ZY07N8-A07_GL	LC FW2 PRT
35	ZY07N8-A07_GL	LC FW3 PRT

36	ZY07N8-A07_GL	LC FW4 PRT
37	5T4_0060	VH DNA
38	5T4_0060	VH PRT
39	HC 5T4_0060	HC CDR1 PRT
40	HC 5T4_0060	HC CDR2 PRT
41	HC 5T4_0060	HC CDR3 PRT
42	HC 5T4_0060	HC FW1 PRT
43	HC 5T4_0060	HC FW2 PRT
44	HC 5T4_0060	HC FW3 PRT
45	HC 5T4_0060	HC FW4 PRT
46	5T4_0060	VL DNA
47	5T4_0060	VL PRT
48	5T4_0060	LC CDR1 PRT
49	5T4_0060	LC CDR2 PRT
50	5T4_0060	LC CDR3 PRT
51	5T4_0060	LC FW1 PRT
52	5T4_0060	LC FW2 PRT
53	5T4_0060	LC FW3 PRT
54	5T4_0060	LC FW4 PRT
55	5T4_0065	VH DNA
56	5T4_0065	VH PRT

57	HC 5T4_0065	HC CDR1 PRT
58	HC 5T4_0065	HC CDR2 PRT
59	HC 5T4_0065	HC CDR3 PRT
60	HC 5T4_0065	HC FW1 PRT
61	HC 5T4_0065	HC FW2 PRT
62	HC 5T4_0065	HC FW3 PRT
63	HC 5T4_0065	HC FW4 PRT
64	5T4_0065	VL DNA
65	5T4_0065	VL PRT
66	5T4_0065	LC CDR1 PRT
67	5T4_0065	LC CDR2 PRT
68	5T4_0065	LC CDR3 PRT
69	5T4_0065	LC FW1 PRT
70	5T4_0065	LC FW2 PRT
71	5T4_0065	LC FW3 PRT
72	5T4_0065	LC FW4 PRT
73	5T4_0068	VH DNA
74	5T4_0068	VH PRT
75	5T4_0068	HC CDR1 PRT
76	5T4_0068	HC CDR2 PRT
77	5T4_0068	HC CDR3 PRT

78	5T4_0068	HC FW1 PRT
79	5T4_0068	HC FW2 PRT
80	5T4_0068	HC FW3 PRT
81	5T4_0068	HC FW4 PRT
82	5T4_0068	VL DNA
83	5T4_0068	VL PRT
84	5T4_0068	LC CDR1 PRT
85	5T4_0068	LC CDR2 PRT
86	5T4_0068	LC CDR3 PRT
87	5T4_0068	LC FW1 PRT
88	5T4_0068	LC FW2 PRT
89	5T4_0068	LC FW3 PRT
90	5T4_0068	LC FW4 PRT
91	5T4_0069	VH DNA
92	5T4_0069	VH PRT
93	5T4_0069	HC CDR1 PRT
94	5T4_0069	HC CDR2 PRT
95	5T4_0069	HC CDR3 PRT
96	5T4_0069	HC FW1 PRT
97	5T4_0069	HC FW2 PRT
98	5T4_0069	HC FW3 PRT

99	5T4_0069	HC FW4 PRT
100	5T4_0069	VL DNA
101	5T4_0069	VL PRT
102	5T4_0069	LC CDR1 PRT
103	5T4_0069	LC CDR2 PRT
104	5T4_0069	LC CDR3 PRT
105	5T4_0069	LC FW1 PRT
106	5T4_0069	LC FW2 PRT
107	5T4_0069	LC FW3 PRT
108	5T4_0069	LC FW4 PRT
109	5T4_0112	VH DNA
110	5T4_0112	VH PRT
111	5T4_0112	HC CDR1 PRT
112	5T4_0112	HC CDR2 PRT
113	5T4_0112	HC CDR3 PRT
114	5T4_0112	HC FW1 PRT
115	5T4_0112	HC FW2 PRT
116	5T4_0112	HC FW3 PRT
117	5T4_0112	HC FW4 PRT
118	5T4_0112	VL DNA
119	5T4_0112	VL PRT

120	5T4_0112	LC CDR1 PRT
121	5T4_0112	LC CDR2 PRT
122	5T4_0112	LC CDR3 PRT
123	5T4_0112	LC FW1 PRT
124	5T4_0112	LC FW2 PRT
125	5T4_0112	LC FW3 PRT
126	5T4_0112	LC FW4 PRT
127	5T4_0113	VH DNA
128	5T4_0113	VH PRT
129	5T4_0113	HC CDR1 PRT
130	5T4_0113	HC CDR2 PRT
131	5T4_0113	HC CDR3 PRT
132	5T4_0113	HC FW1 PRT
133	5T4_0113	HC FW2 PRT
134	5T4_0113	HC FW3 PRT
135	5T4_0113	HC FW4 PRT
136	5T4_0113	VL DNA
137	5T4_0113	VL PRT
138	5T4_0113	LC CDR1 PRT
139	5T4_0113	LC CDR2 PRT
140	5T4_0113	LC CDR3 PRT

141	5T4_0113	LC FW1 PRT
142	5T4_0113	LC FW2 PRT
143	5T4_0113	LC FW3 PRT
144	5T4_0113	LC FW4 PRT
145	EVQLVESGGGLVKPGGSLRLSCAASGX$_1$X$_2$X$_3$X$_4$	HC FW1 consensus sequence
146	GX$_1$X$_2$X$_3$X$_4$X$_5$X$_6$DX$_7$X$_8$X$_9$ wherein X$_1$=F, D or E, X$_2$=T, W or G, X$_3$=F, A S or T, X$_4$= S, A, Q, H, T, G or K, X$_5$=N, T, Q, R, A, L, P or S, X$_6$=Q, Y, A, L, F, M or S, X$_7$=P, W, Q, or L, X$_8$=M, L, I, G or V, X$_9$=H, S or T,	a first HC CDR1 sequence
147	X$_1$X$_2$X$_3$X$_4$X$_5$X$_6$DX$_7$X$_8$X$_9$X$_{10}$X$_{11}$SAAPVKG, wherein X$_1$=R, A, or Q, X$_2$=I, V, L, M, or Q, X$_3$=R or K, X$_4$=A, S, or G, X$_5$=Q, K, E, S, V, I, H, L, R, or Y,	a first HC CDR2 consensus sequence

	X_6=A, Q, R, L, M, X_7=G, N, E, L, X_8=G, A, D, E, Q, X_9=T, E, Q, R, V, W, X_{10}=T, Q, V, I, P, and X_{11}=D, Q, V, F, W	
148	VDRRNYYGMDV	a first HC CDR2 consensus sequence
149	RASQGIRNDLG	
150	$X_1X_2X_3X_4X_5X_6$S, wherein X_1=A or N, X_2=A, G, S, T, or V, X_3=S, W, D, G, N, Q, H, or T, X_4=R, Q, T, S, H, or K, X_5=R, L, P, W, H, K, M, N, Q, S, T, or V, and X_6=D, M, S, or V	a first LC CDR2 consensus sequence
151	X_1Q$X_2X_3X_4X_5$PWX_6, wherein X_1=L or V, X_2=Q, M, or L, X_3=R, K, Y, F, N, A, H, T, or W, X_4=R, K, A, N, Q, L, M, S, or T, X_5=M, T, V, A, I, Q, Y, L, P, K, R, or S, and X_6=T, R, M, V, A, K, or S	a first LC CDR3 consensus sequence

153	$X_1IX_2AX_3X_4DGGTTDSAAPVKG$, wherein X_1=R or Q, X_2=R or K, X_3=Q, S, V, and X_4=R, Q, A	a second HC CDR2 consensus sequence
156	$X_1X_2X_3X_4X_5X_6S$, wherein X_1=A or N, X_2=G, A, or S, X_3=W, S, or G, X_4=Q, T, or R, X_5=R, L, or P, and X_6=D, M, S, or V	a second LC CDR2 consensus sequence
157	$LQX_1X_2X_3X_4PWX_5$, wherein X_1=Q, M, L, X_2=R, Y, K, F, H, N, X_3=R, K, N, A, X_4=M, T, V, I, P, and X_5=T, M, V	a second LC CDR3 consensus sequence
158	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG	Fc wild type sequence

	VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPGK	
159	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSCV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPGK	Fc 239iCys
160	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLC LSPGK	Fc S442C

161	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPSCV FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLC LSPGK	Fc 239iCys & S442C
162	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSW NSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC NVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELLGGPCVF LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS LSPGK	Fc S239C

DETAILED DESCRIPTION OF THE INVENTION

[064] The present disclosure provides optimized anti-5T4 antibodies, as well as antibody-drug conjugates (ADC) derived from such optimized antibodies. Related polynucleotides, vectors, cells, and pharmaceutical compositions comprising such optimized anti-5T4 antibodies, or the ADC are also provided. The anti-5T4 antibodies may also be conjugated to imaging agents. Further provided are methods of making such optimized anti-5T4 antibodies as well as methods of making antibody-drug conjugates (ADC) derived from such optimized antibodies. Also provided are methods of using the anti-5T4 ADCs derived from such optimized anti-5T4 antibodies, for example, methods of treating cancer in a subject in need thereof, are further provided

[065] In order that the present invention can be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

Definitions

[066] Before describing the present invention in detail, it is to be understood that this invention is not limited to specific compositions or process steps, as such can vary. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. The terms "a" (or "an"), as well as the terms "one or more," and "at least one" can be used interchangeably herein.

[067] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[068] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[069] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects , which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[070] It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[071] Amino acids are referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, are referred to by their commonly accepted single-letter codes.

[072] As used herein, the term "tubulysin" refers both collectively and individually to the naturally occurring tubulysins, and the analogs and derivatives of tubulysins. Illustrative examples of tubulysins are disclosed, for example, in WO2004005326A2, WO2012019123A1,

WO2009134279A1, WO2009055562A1, WO2004005327A1, US7754885, US20100240701, US7816377, US20110021568, and US20110263650. It is to be understood that such derivatives include, for example, tubulysin prodrugs or tubulysins that include one or more protection or protecting groups.

[073] The terms "antibody" or "immunoglobulin," as used interchangeably herein, include whole antibodies and any antigen binding fragment or single chains thereof.

[074] A typical antibody comprises at least two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2, and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed Complementarity Determining Regions (CDR), interspersed with regions that are more conserved, termed framework regions (FW). Each VH and VL is composed of three CDRs and four FWs, arranged from amino-terminus to carboxy-terminus in the following order: FW1, CDR1, FW2, CDR2, FW3, CDR3, FW4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies can mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (C1q) of the classical complement system. Exemplary antibodies of the present disclosure include typical antibodies, scFvs, and combinations thereof where, for example, an scFv is covalently linked (for example, via peptidic bonds or via a

chemical linker) to the N-terminus of either the heavy chain and/or the light chain of a typical antibody, or intercalated in the heavy chain and/or the light chain of a typical antibody.

[075] The term "antibody" means an immunoglobulin molecule that recognizes and specifically binds to a target, such as a protein, polypeptide, peptide, carbohydrate, polynucleotide, lipid, or combinations of the foregoing through at least one antigen recognition site within the variable region of the immunoglobulin molecule. As used herein, the term "antibody" encompasses intact polyclonal antibodies, intact monoclonal antibodies, antibody fragments (such as Fab, Fab', F(ab')2, and Fv fragments), single chain variable fragment (scFv), disulfide stabilized scFvs, multispecific antibodies such as bispecific antibodies generated from at least two intact antibodies and/or antigen binding fragments thereof, chimeric antibodies, humanized antibodies, human antibodies, fusion proteins comprising an antigen determination portion of an antibody, and any other modified immunoglobulin molecule comprising an antigen recognition site so long as the antibodies exhibit the desired biological activity. An antibody can be of any the five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses (isotypes) thereof (*e.g.* IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu, respectively. The different classes of immunoglobulins have different and well known subunit structures and three-dimensional configurations. Antibodies can be naked or conjugated to other molecules such as toxins, radioisotopes, etc. to form Antibody Drug Conjugates (ADC).

[076] The terms "anti-5T4 antibody" or "anti-5T4" refers to an antibody that is capable of binding 5T4 with sufficient affinity such that the antibody is useful as a therapeutic agent or diagnostic reagent in targeting 5T4. The extent of binding of an anti-5T4 antibody to an unrelated, non-5T4 protein is less than about 10% of the binding of the antibody to 5T$ as

measured, *e.g.*, by a radioimmunoassay (RIA), BIACORE™ (using recombinant 5T4 as the analyte and antibody as the ligand, or *vice versa*), or other binding assays known in the art. In certain aspects, an antibody that binds to 5T4 has a dissociation constant (K_D) of ≤1 μM, ≤100 nM, ≤10 nM, ≤1 nM, ≤0.1 nM, ≤10 pM, ≤1 pM, or ≤0.1 pM.

[077] The terms "antigen binding fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. It is known in the art that the antigen binding function of an antibody can be performed by fragments of a full-length antibody. Examples of antibody fragments include, but are not limited to Fab, Fab', F(ab')2, and Fv fragments, linear antibodies, single chain antibodies, and multispecific antibodies formed from antibody fragments.

[078] A "monoclonal antibody" refers to a homogeneous antibody population involved in the highly specific recognition and binding of a single antigenic determinant, or epitope. This is in contrast to polyclonal antibodies that typically include different antibodies directed against different antigenic determinants.

[079] The term "monoclonal antibody" encompasses both intact and full-length monoclonal antibodies as well as antibody fragments (such as Fab, Fab', F(ab')2, Fv), single chain variable fragments (scFv), fusion proteins comprising an antibody portion, and any other modified immunoglobulin molecule comprising an antigen recognition site. Furthermore, "monoclonal antibody" refers to such antibodies made in any number of ways including, but not limited to, by hybridoma, phage selection, recombinant expression, and transgenic animals.

[080] The term "humanized antibody" refers to an antibody derived from a non-human (*e.g.*, murine) immunoglobulin, which has been engineered to contain minimal non-human (*e.g.*, murine) sequences. Typically, humanized antibodies are human immunoglobulins in which

residues from the complementary determining region (CDR) are replaced by residues from the CDR of a non-human species (*e.g.*, mouse, rat, rabbit, or hamster) that have the desired specificity, affinity, and capability (Jones *et al.*, 1986, *Nature*, 321:522-525; Riechmann *et al.*, 1988, *Nature*, 332:323-327; Verhoeyen *et al.*, 1988, *Science*, 239:1534-1536). In some instances, the Fv framework region (FW) residues of a human immunoglobulin are replaced with the corresponding residues in an antibody from a non-human species that has the desired specificity, affinity, and capability.

[081] The humanized antibody can be further modified by the substitution of additional residues either in the Fv framework region and/or within the replaced non-human residues to refine and optimize antibody specificity, affinity, and/or capability. In general, the humanized antibody will comprise substantially all of at least one, and typically two or three, variable domains containing all or substantially all of the CDR regions that correspond to the non-human immunoglobulin whereas all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody can also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Examples of methods used to generate humanized antibodies are described in U.S. Pat. Nos. 5,225,539 or 5,639,641.

[082] A "variable region" of an antibody refers to the variable region of the antibody light chain or the variable region of the antibody heavy chain, either alone or in combination. The variable regions of the heavy and light chain each consist of four framework regions (FW) connected by three complementarity determining regions (CDRs) also known as hypervariable regions. The CDRs in each chain are held together in close proximity by the FW regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of

antibodies. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (*i.e.*, Kabat *et al.* Sequences of Proteins of Immunological Interest, (5th ed., 1991, National Institutes of Health, Bethesda Md.)); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Al-lazikani *et al.* (1997) J. *Molec. Biol.* 273:927-948)). In addition, combinations of these two approaches are sometimes used in the art to determine CDRs.

[083] The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (*e.g,*, Kabat *et al.*, Sequences of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)).

[084] The amino acid position numbering as in Kabat, refers to the numbering system used for heavy chain variable domains or light chain variable domains of the compilation of antibodies in Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Using this numbering system, the actual linear amino acid sequence can contain fewer or additional amino acids corresponding to a shortening of, or insertion into, a FW or CDR of the variable domain. For example, a heavy chain variable domain can include a single amino acid insert (residue 52a according to Kabat) after residue 52 of H2 and inserted residues (*e.g.*, residues 82a, 82b, and 82c, *etc.* according to Kabat) after heavy chain FW residue 82.

[085] TABLE 1

Loop	Kabat	AbM	Chothia
L1	L24-L34	L24-L34	L24-L34
L2	L50-L56	L50-L56	L50-L56
L3	L89-L97	L89-L97	L89-L97
H1	H31-H35B	H26-H35B (Kabat Numbering)	H26-H32..34
H1	H31-H35	H26-H35 (Chothia Numbering)	H26-H32
H2	H50-H65	H50-H58	H52-H56
H3	H95-H102	H95-H102	H95-H102

[086]

[087] The Kabat numbering of residues can be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence. Chothia refers instead to the location of the structural loops (Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987)). The end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software.

[088] IMGT (ImMunoGeneTics) also provides a numbering system for the immunoglobulin variable regions, including the CDRs. See *e.g.*, Lefranc, M.P. *et al.*, Dev. Comp. Immunol. 27: 55-77(2003), which is herein incorporated by reference. The IMGT numbering system was based on an alignment of more than 5,000 sequences, structural data, and characterization of hypervariable loops and allows for easy comparison of the variable and CDR regions for all species. According to the IMGT numbering schema VH-CDR1 is at positions 26

to 35, VH-CDR2 is at positions 51 to 57, VH-CDR3 is at positions 93 to 102, VL-CDR1 is at positions 27 to 32, VL-CDR2 is at positions 50 to 52, and VL-CDR3 is at positions 89 to 97.

[089] As used herein the Fc region includes the polypeptides comprising the constant region of an antibody excluding the first constant region immunoglobulin domain. Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc can include the J chain. For IgG, Fc comprises immunoglobulin domains Cgamma2 and Cgamma3 (Cγ2 and Cγ3) and the hinge between Cgamma1 (Cγ1) and Cgamma2 (Cγ2). Although the boundaries of the Fc region can vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as set forth in Kabat (Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). Fc can refer to this region in isolation, or this region in the context of an antibody, antibody fragment, or Fc fusion protein. Polymorphisms have been observed at a number of different Fc positions, including but not limited to positions 270, 272, 312, 315, 356, and 358 as numbered by the EU index, and thus slight differences between the presented sequence and sequences in the prior art may exist.

[090] The term "chimeric antibodies" refers to antibodies wherein the amino acid sequence of the immunoglobulin molecule is derived from two or more species. Typically, the variable region of both light and heavy chains corresponds to the variable region of antibodies derived from one species of mammals (*e.g.*, mouse, rat, rabbit, etc.) with the desired specificity, affinity, and capability while the constant regions are homologous to the sequences in antibodies derived from another (usually human) to avoid eliciting an immune response in that species.

[091] "Binding affinity" generally refers to the strength of the sum total of non-covalent 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. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present disclosure.

[092] "Potency" is normally expressed as an IC_{50} value, in nM unless otherwise stated. IC_{50} is the median inhibitory concentration of an antigen-binding molecule. In functional assays, IC_{50} is the concentration that reduces a biological response by 50% of its maximum. In ligand-binding studies, IC_{50} is the concentration that reduces receptor binding by 50% of maximal specific binding level. IC_{50} can be calculated by any number of means known in the art. Improvement in potency can be determined by measuring against a parent antibody,

[093] A polypeptide, antibody, polynucleotide, vector, cell, or composition which is "isolated" is a polypeptide, antibody, polynucleotide, vector, cell, or composition which is in a form not found in nature. Isolated polypeptides, antibodies, polynucleotides, vectors, cells or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In some aspects, an antibody, polynucleotide, vector, cell, or composition which is isolated is substantially pure.

[094] The term "subject" refers to any animal (*e.g.*, a mammal), including, but not limited to humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

[095] The term "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of the active ingredient (*e.g.*, an anti-5T4 ADC disclosed herein) to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the composition would be administered. Such composition can be sterile.

[096] An "effective amount" of an anti-5T4 ADC as disclosed herein is an amount sufficient to carry out a specifically stated purpose. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose.

[097] The term "therapeutically effective amount" refers to an amount of an anti-5T4 binding molecule disclosed herein or other drug effective to "treat" a disease or disorder in a subject or mammal.

[098] The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to an anti-5T4 binding molecule disclosed herein so as to generate a "labeled" anti-5T4 binding molecule . The label can be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, can catalyze chemical alteration of a substrate compound or composition which is detectable.

[099] Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to both (1) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder and (2) prophylactic or

preventative measures that prevent and/or slow the development of a targeted pathologic condition or disorder. Thus, those in need of treatment include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. In certain aspects, a subject is successfully "treated" for cancer according to the methods of the present disclosure if the patient shows, *e.g.*, total, partial, or transient remission of a certain type of cancer.

[0100] The terms "cancer", "tumor", "cancerous", and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancers include but are not limited to, carcinoma including adenocarcinomas, lymphomas, blastomas, melanomas, sarcomas, and leukemias. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, glioma, cervical cancer, ovarian cancer, liver cancer such as hepatic carcinoma and hepatoma, bladder cancer, breast cancer (including hormonally mediated breast cancer, see, *e.g.*, Innes *et al.* (2006) Br. J. Cancer 94:1057-1065), colon cancer, colorectal cancer, endometrial carcinoma, myeloma (such as multiple myeloma), salivary gland carcinoma, kidney cancer such as renal cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, various types of head and neck cancer and cancers of mucinous origins, such as, mucinous ovarian cancer, cholangiocarcinoma (liver) and renal papillary carcinoma.

[0101] As used herein, the term "carcinomas" refers to cancers of epithelial cells, which are cells that cover the surface of the body, produce hormones, and make up glands. Examples of carcinomas are cancers of the skin, lung, colon, stomach, breast, prostate and thyroid gland.

[0102] "Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and their analogs. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0103] The term "vector" means a construct, which is capable of delivering, and in some aspects, expressing, one or more gene(s) or sequence(s) of interest in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells.

[0104] The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer can be linear or branched, it can comprise modified amino acids, and it can be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that, because the polypeptides of the instant

disclosure are based upon antibodies, in certain aspects, the polypeptides can occur as single chains or associated chains.

[0105] A "recombinant" polypeptide or protein refers to a polypeptide or protein produced via recombinant DNA technology. Recombinantly produced polypeptides and proteins expressed in engineered host cells are considered isolated for the purpose of the invention, as are native or recombinant polypeptides which have been separated, fractionated, or partially or substantially purified by any suitable technique. The polypeptides disclosed herein can be recombinantly produced using methods known in the art. Alternatively, the proteins and peptides disclosed herein can be chemically synthesized.

[0106] The term "amino acid substitution" refers to replacing an amino acid residue present in a parent sequence with another amino acid residue. An amino acid can be substituted in a parent sequence, for example, via chemical peptide synthesis or through recombinant methods known in the art. Accordingly, references to a "substitution at position X" or "substitution at position X" refer to the substitution of an amino acid present at position X with an alternative amino acid residue. Substitution patterns can described according to the schema AXY, wherein A is the single letter code corresponding to the amino acid naturally present at position X, and A is the substituting amino acid residue. Accordingly, L234F would refer to the substitution of the leucine amino acid (L) at position 234 with a phenylalanine (F).

[0107] A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side

chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, if an amino acid in a polypeptide is replaced with another amino acid from the same side chain family, the substitution is considered to be conservative. In another aspect, a string of amino acids can be conservatively replaced with a structurally similar string that differs in order and/or composition of side chain family members.

[0108] Non-conservative substitutions include those in which (i) a residue having an electropositive side chain (*e.g.*, Arg, His or Lys) is substituted for, or by, an electronegative residue (*e.g.*, Glu or Asp), (ii) a hydrophilic residue (*e.g.*, Ser or Thr) is substituted for, or by, a hydrophobic residue (*e.g.*, Ala, Leu, Ile, Phe or Val), (iii) a cysteine or proline is substituted for, or by, any other residue, or (iv) a residue having a bulky hydrophobic or aromatic side chain (*e.g.*, Val, His, Ile or Trp) is substituted for, or by, one having a smaller side chain (*e.g.*, Ala, Ser) or no side chain (*e.g.*, Gly).

[0109] Other substitutions can be readily identified by workers of ordinary skill. For example, for the amino acid alanine, a substitution can be taken from any one of D-alanine, glycine, beta-alanine, L-cysteine and D-cysteine. For lysine, a replacement can be any one of D-lysine, arginine, D-arginine, homo-arginine, methionine, D-methionine, omithine, or D-ornithine. Generally, substitutions in functionally important regions that can be expected to induce changes in the properties of isolated polypeptides are those in which: (i) a polar residue, *e.g.*, serine or threonine, is substituted for (or by) a hydrophobic residue, *e.g.*, leucine, isoleucine, phenylalanine, or alanine; (ii) a cysteine residue is substituted for (or by) any other residue; (iii) a residue having an electropositive side chain, *e.g.*, lysine, arginine or histidine, is substituted for

(or by) a residue having an electronegative side chain, *e.g.*, glutamic acid or aspartic acid; or (iv) a residue having a bulky side chain, *e.g.*, phenylalanine, is substituted for (or by) one not having such a side chain, *e.g.*, glycine. The likelihood that one of the foregoing non-conservative substitutions may alter functional properties of the protein is also correlated to the position of the substitution with respect to functionally important regions of the protein: some non-conservative substitutions may accordingly have little or no effect on biological properties.

[0110] The term "amino acid insertion" refers to introducing a new amino acid residue between two amino acid residues present in the parent sequence. An amino acid can be inserted in a parent sequence, for example, via chemical peptide synthesis or through recombinant methods known in the art. Accordingly as used herein, the phrase "insertion between positions X and Y," wherein X and Y correspond to amino acid positions (e.g., a cysteine amino acid insertion between positions 239 and 240), refers to the insertion of an amino acid between the X and Y positions, and also to the insertion in a nucleic acid sequence of a codon encoding an amino acid between the codons encoding the amino acids at positions X and Y. Insertion patterns can be described according to the schema AX-ins, wherein A is the single letter code corresponding to the amino acid being inserted, and X is the position preceeding the insertion. Accordingly, C239-ins would refer to the insertion of a cysteine amino acid (C) after position 239 (i.e., an intertion between position 239 and 240]]

[0111] The term "percent sequence identity" between two polypeptide or polynucleotide sequences refers to the number of identical matched positions shared by the sequences over a comparison window, taking into account additions or deletions (*i.e.*, gaps) that must be introduced for optimal alignment of the two sequences. A matched position is any position where an identical nucleotide or amino acid is presented in both the target and reference sequence. Gaps

presented in the target sequence are not counted since gaps are not nucleotides or amino acids. Likewise, gaps presented in the reference sequence are not counted since target sequence nucleotides or amino acids are counted, not nucleotides or amino acids from the reference sequence.

[0112] The percentage of sequence identity is calculated by determining the number of positions at which the identical amino-acid residue or nucleic acid base occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. The comparison of sequences and determination of percent sequence identity between two sequences may be accomplished using readily available software both for online use and for download. Suitable software programs are available from various sources, and for alignment of both protein and nucleotide sequences. One suitable program to determine percent sequence identity is bl2seq, part of the BLAST suite of program available from the U.S. government's National Center for Biotechnology Information BLAST web site (blast.ncbi.nlm.nih.gov). Bl2seq performs a comparison between two sequences using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. Other suitable programs are, *e.g.*, Needle, Stretcher, Water, or Matcher, part of the EMBOSS suite of bioinformatics programs and also available from the European Bioinformatics Institute (EBI) at www.ebi.ac.uk/Tools/psa.

[0113] Different regions within a single polynucleotide or polypeptide target sequence that aligns with a polynucleotide or polypeptide reference sequence can each have their own percent sequence identity. It is noted that the percent sequence identity value is rounded to the nearest tenth. For example, 80.11, 80.12, 80.13, and 80.14 are rounded down to 80.1, while

80.15, 80.16, 80.17, 80.18, and 80.19 are rounded up to 80.2. It also is noted that the length value will always be an integer.

[0114] In certain aspects, the percentage identity "X" of a first amino acid sequence to a second sequence amino acid is calculated as 100 x (Y/Z), where Y is the number of amino acid residues scored as identical matches in the alignment of the first and second sequences (as aligned by visual inspection or a particular sequence alignment program) and Z is the total number of residues in the second sequence. If the length of a first sequence is longer than the second sequence, the percent identity of the first sequence to the second sequence will be higher than the percent identity of the second sequence to the first sequence.

[0115] One skilled in the art will appreciate that the generation of a sequence alignment for the calculation of a percent sequence identity is not limited to binary sequence-sequence comparisons exclusively driven by primary sequence data. Sequence alignments can be derived from multiple sequence alignments. One suitable program to generate multiple sequence alignments is ClustalW2, available from www.clustal.org. Another suitable program is MUSCLE, available from www.drive5.com/muscle/. ClustalW2 and MUSCLE are alternatively available, *e.g.*, from the EBI.

[0116] It will also be appreciated that sequence alignments can be generated by integrating sequence data with data from heterogeneous sources such as structural data (*e.g.*, crystallographic protein structures), functional data (*e.g.*, location of mutations), or phylogenetic data. A suitable program that integrates heterogeneous data to generate a multiple sequence alignment is T-Coffee, available at www.tcoffee.org, and alternatively available, *e.g.*, from the EBI. It will also be appreciated that the final alignment used to calculate percent sequence identity may be curated either automatically or manually.

[0117] The term "pharmaceutically acceptable cations" refers to pharmaceutically acceptable monovalent and divalent cations, such as those discussed in Berge, et al., J. Pharm. Sci., 66, 1-19 (1977), which is incorporated herein by reference. The pharmaceutically acceptable cation may be inorganic or organic. Examples of pharmaceutically acceptable monovalent inorganic cations include, but are not limited to, alkali metal ions such as Na^+ and K^+. Examples of pharmaceutically acceptable divalent inorganic cations include, but are not limited to, alkaline earth cations such as Ca^{2+} and Mg^{2+}. Examples of pharmaceutically acceptable organic cations include, but are not limited to, ammonium ion (i.e. NH_4^+) and substituted ammonium ions (e.g. NH_3R^+, $NH_2R_2^+$, NHR_3^+, NR_4^+). Examples of some suitable substituted ammonium ions are those derived from: ethylamine, diethylamine, dicyclohexylamine, triethylamine, butylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine, benzylamine, phenylbenzylamine, choline, meglumine, and tromethamine, as well as amino acids, such as lysine and arginine. An example of a common quaternary ammonium ion is $N(CH_3)_4^+$.

[0118] The phrase "optionally substituted" as used herein, pertains to a parent group which may be unsubstituted or which may be substituted.

[0119] Unless otherwise specified, the term "substituted" as used herein in the context of modfications to the chemical structure of the cytotoxic agents, i.e. tubulysins or pyrrolobenzodiazepines, pertains to a parent group which bears one or more substituents. The term "substituent" is used herein in the conventional sense and refers to a chemical moiety which is covalently attached to, or if appropriate, fused to, a parent group. A wide variety of substituents are well known, and methods for their formation and introduction into a variety of parent groups are also well known.

[0120] Examples of chemical substituents are described in more detail below.

[0121] The term "C_{1-12} alkyl" as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a carbon atom of a hydrocarbon compound having from 1 to 12 carbon atoms, which may be aliphatic or alicyclic, and which may be saturated or unsaturated (e.g. partially unsaturated, fully unsaturated). The term "C_{1-4} alkyl" as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a carbon atom of a hydrocarbon compound having from 1 to 4 carbon atoms, which may be aliphatic or alicyclic, and which may be saturated or unsaturated (e.g. partially unsaturated, fully unsaturated). Thus, the term "alkyl" includes the sub-classes alkenyl, alkynyl, cycloalkyl, etc., discussed below.

[0122] Examples of saturated alkyl groups include, but are not limited to, methyl (C_1), ethyl (C_2), propyl (C_3), butyl (C_4), pentyl (C_5), hexyl (C_6) and heptyl (C_7).

[0123] Examples of saturated linear alkyl groups include, but are not limited to, methyl (C_1), ethyl (C_2), n-propyl (C_3), n-butyl (C_4), n-pentyl (amyl) (C_5), n-hexyl (C_6) and n-heptyl (C_7).

[0124] Examples of saturated branched alkyl groups include iso-propyl (C_3), iso-butyl (C_4), sec-butyl (C_4), tert-butyl (C_4), iso-pentyl (C_5), and neo-pentyl (C_5).

[0125] The term "C_{2-12} alkenyl" as used herein, pertains to an alkyl group having one or more carbon-carbon double bonds.

[0126] Examples of unsaturated alkenyl groups include, but are not limited to, ethenyl (vinyl, -CH=CH_2), 1-propenyl (-CH=CH-CH_3), 2-propenyl (allyl, -CH-CH=CH_2), isopropenyl (1-methylvinyl, -C(CH_3)=CH_2), butenyl (C_4), pentenyl (C_5), and hexenyl (C_6).

[0127] The term "C_{2-12} alkynyl" as used herein, pertains to an alkyl group having one or more carbon-carbon triple bonds.

[0128] Examples of unsaturated alkynyl groups include, but are not limited to, ethynyl (-C≡CH) and 2-propynyl (propargyl, -CH_2-C≡CH).

[0129] The term "C_{3-12} cycloalkyl" as used herein, pertains to an alkyl group which is also a cyclyl group; that is, a monovalent moiety obtained by removing a hydrogen atom from an alicyclic ring atom of a cyclic hydrocarbon (carbocyclic) compound, which moiety has from 3 to 7 carbon atoms, including from 3 to 7 ring atoms.

[0130] Examples of cycloalkyl groups include, but are not limited to, those derived from:

saturated monocyclic hydrocarbon compounds:

cyclopropane (C_3), cyclobutane (C_4), cyclopentane (C_5), cyclohexane (C_6), cycloheptane (C_7), methylcyclopropane (C_4), dimethylcyclopropane (C_5), methylcyclobutane (C_5), dimethylcyclobutane (C_6), methylcyclopentane (C_6), dimethylcyclopentane (C_7) and methylcyclohexane (C_7);

unsaturated monocyclic hydrocarbon compounds:

cyclopropene (C_3), cyclobutene (C_4), cyclopentene (C_5), cyclohexene (C_6), methylcyclopropene (C_4), dimethylcyclopropene (C_5), methylcyclobutene (C_5), dimethylcyclobutene (C_6), methylcyclopentene (C_6), dimethylcyclopentene (C_7) and methylcyclohexene (C_7); and

saturated polycyclic hydrocarbon compounds:

norcarane (C_7), norpinane (C_7), norbornane (C_7).

C_{3-7} heterocyclyl: The term "C_{3-7} heterocyclyl" as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from a ring atom of a monocyclic heterocyclic compound, which moiety has from 3 to 7 ring atoms, of which from 1 to 4 are ring heteroatoms.

[0131] In this context, the prefixes (e.g. C_{3-7}, C_{5-6}, etc.) denote the number of ring atoms, or range of number of ring atoms, whether carbon atoms or heteroatoms. For example, the term "C_{5-6}heterocyclyl", as used herein, pertains to a heterocyclyl group having 5 or 6 ring atoms.

[0132] Examples of monocyclic heterocyclyl groups include, but are not limited to, those derived from:

N_1: aziridine (C_3), azetidine (C_4), pyrrolidine (tetrahydropyrrole) (C_5), pyrroline (e.g., 3-pyrroline, 2,5-dihydropyrrole) (C_5), 2H-pyrrole or 3H-pyrrole (isopyrrole, isoazole) (C_5), piperidine (C_6), dihydropyridine (C_6), tetrahydropyridine (C_6), azepine (C_7);

O_1: oxirane (C_3), oxetane (C_4), oxolane (tetrahydrofuran) (C_5), oxole (dihydrofuran) (C_5), oxane (tetrahydropyran) (C_6), dihydropyran (C_6), pyran (C_6), oxepin (C_7);

S_1: thiirane (C_3), thietane (C_4), thiolane (tetrahydrothiophene) (C_5), thiane (tetrahydrothiopyran) (C_6), thiepane (C_7);

O_2: dioxolane (C_5), dioxane (C_6), and dioxepane (C_7);

O_3: trioxane (C_6);

N_2: imidazolidine (C_5), pyrazolidine (diazolidine) (C_5), imidazoline (C_5), pyrazoline (dihydropyrazole) (C_5), piperazine (C_6);

N_1O_1: tetrahydrooxazole (C_5), dihydrooxazole (C_5), tetrahydroisoxazole (C_5), dihydroisoxazole (C_5), morpholine (C_6), tetrahydrooxazine (C_6), dihydrooxazine (C_6), oxazine (C_6);

N_1S_1: thiazoline (C_5), thiazolidine (C_5), thiomorpholine (C_6);

N_2O_1: oxadiazine (C_6);

O_1S_1: oxathiole (C_5) and oxathiane (thioxane) (C_6); and,

$N_1O_1S_1$: oxathiazine (C_6).

[0133] The term "C_{5-10} aryl", as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from an aromatic ring atom of an aromatic compound, which moiety has from 5 to 10 ring atoms. The term "C_{5-7} aryl", as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from an aromatic ring atom of an aromatic compound, which moiety has from 5 to 7 ring atoms and the term "C_{5-10} aryl", as used herein, pertains to a monovalent moiety obtained by removing a hydrogen atom from an aromatic ring atom of an aromatic compound, which moiety has from 5 to 10 ring atoms. Preferably, each ring has from 5 to 7 ring atoms.

[0134] In this context, the prefixes (e.g. C_{3-10}, C_{5-7}, C_{5-6}, C_{5-10}, etc.) denote the number of ring atoms, or range of number of ring atoms, whether carbon atoms or heteroatoms. For example, the term "C_{5-6} aryl" as used herein, pertains to an aryl group having 5 or 6 ring atoms.

[0135] The ring atoms may be all carbon atoms, as in "carboaryl groups".

[0136] Examples of carboaryl groups include, but are not limited to, those derived from benzene (i.e. phenyl) (C_6), naphthalene (C_{10}), azulene (C_{10}), anthracene (C_{14}), phenanthrene (C_{14}), naphthacene (C_{18}), and pyrene (C_{16}).

[0137] Examples of aryl groups which comprise fused rings, at least one of which is an aromatic ring, include, but are not limited to, groups derived from indane (e.g. 2,3-dihydro-1H-indene) (C_9), indene (C_9), isoindene (C_9), and tetraline (1,2,3,4-tetrahydronaphthalene (C_{10}),.

[0138] Alternatively, the ring atoms may include one or more heteroatoms, as in "heteroaryl groups". Examples of monocyclic heteroaryl groups include, but are not limited to, those derived from:

N_1: pyrrole (azole) (C_5), pyridine (azine) (C_6);

O_1: furan (oxole) (C_5);

S_1: thiophene (thiole) (C_5);

N_1O_1: oxazole (C_5), isoxazole (C_5), isoxazine (C_6);

N_2O_1: oxadiazole (furazan) (C_5);

N_3O_1: oxatriazole (C_5);

N_1S_1: thiazole (C_5), isothiazole (C_5);

N_2: imidazole (1,3-diazole) (C_5), pyrazole (1,2-diazole) (C_5), pyridazine (1,2-diazine) (C_6), pyrimidine (1,3-diazine) (C_6) (e.g., cytosine, thymine, uracil), pyrazine (1,4-diazine) (C_6);

N_3: triazole (C_5), triazine (C_6); and,

N_4: tetrazole (C_5).

[0139] Examples of heteroaryl which comprise fused rings, include, but are not limited to:

C_9 (with 2 fused rings) derived from benzofuran (O_1), isobenzofuran (O_1), indole (N_1), isoindole (N_1), indolizine (N_1), indoline (N_1), isoindoline (N_1), purine (N_4) (e.g., adenine, guanine), benzimidazole (N_2), indazole (N_2), benzoxazole (N_1O_1), benzisoxazole (N_1O_1), benzodioxole (O_2), benzofurazan (N_2O_1), benzotriazole (N_3), benzothiofuran (S_1), benzothiazole (N_1S_1), benzothiadiazole (N_2S); and

C_{10} (with 2 fused rings) derived from chromene (O_1), isochromene (O_1), chroman (O_1), isochroman (O_1), benzodioxan (O_2), quinoline (N_1), isoquinoline (N_1), quinolizine (N_1), benzoxazine (N_1O_1), benzodiazine (N_2), pyridopyridine (N_2), quinoxaline (N_2), quinazoline (N_2), cinnoline (N_2), phthalazine (N_2), naphthyridine (N_2), pteridine (N_4).

[0140] The above groups, whether alone or part of another substituent, may themselves optionally be substituted with one or more groups selected from themselves and the additional substituents listed below.

Halo: -F, -Cl, -Br, and -I.

Hydroxy: -OH.

Ether: -OR, wherein R is an ether substituent, for example, a C_{1-7} alkyl group (also referred to as a C_{1-7} alkoxy group, discussed below), a C_{3-20} heterocyclyl group (also referred to as a C_{3-20} heterocyclyloxy group), or a C_{5-20} aryl group (also referred to as a C_{5-20} aryloxy group), preferably a C_{1-7}alkyl group.

Alkoxy: -OR, wherein R is an alkyl group, for example, a C_{1-7} alkyl group. Examples of C_{1-7} alkoxy groups include, but are not limited to, -OMe (methoxy), -OEt (ethoxy), -O(nPr) (n-propoxy), -O(iPr) (isopropoxy), -O(nBu) (n-butoxy), -O(sBu) (sec-butoxy), -O(iBu) (isobutoxy), and -O(tBu) (tert-butoxy).

Formyl (carbaldehyde, carboxaldehyde): -C(=O)H.

Acyl (keto): -C(=O)R, wherein R is an acyl substituent, for example, a C_{1-7} alkyl group (also referred to as C_{1-7} alkylacyl or C_{1-7} alkanoyl), a C_{3-20} heterocyclyl group (also referred to as C_{3-20} heterocyclylacyl), or a C_{5-20} aryl group (also referred to as C_{5-20} arylacyl), preferably a C_{1-7} alkyl group. Examples of acyl groups include, but are not limited to, -C(=O)CH_3 (acetyl), -C(=O)CH_2CH_3 (propionyl), -C(=O)$C(CH_3)_3$ (t-butyryl), and -C(=O)Ph (benzoyl, phenone).

Carboxy (carboxylic acid): -C(=O)OH.

Ester (carboxylate, carboxylic acid ester, oxycarbonyl): -C(=O)OR, wherein R is an ester substituent, for example, a C_{1-7} alkyl group, a C_{3-20} heterocyclyl group, or a C_{5-20} aryl group,

preferably a C_{1-7} alkyl group. Examples of ester groups include, but are not limited to, $-C(=O)OCH_3$, $-C(=O)OCH_2CH_3$, $-C(=O)OC(CH_3)_3$, and -C(=O)OPh.

Amino: -NR1R2, wherein R1 and R2 are independently amino substituents, for example, hydrogen, a C1-7 alkyl group (also referred to as C1-7 alkylamino or di-C1-7 alkylamino), a C3-20 heterocyclyl group, or a C5-20 aryl group, preferably H or a C1-7 alkyl group, or, in the case of a "cyclic" amino group, R1 and R2, taken together with the nitrogen atom to which they are attached, form a heterocyclic ring having from 4 to 8 ring atoms. Amino groups may be primary (-NH2), secondary (-NHR1), or tertiary (-NHR1R2), and in cationic form, may be quaternary (-+NR1R2R3). Examples of amino groups include, but are not limited to, -NH2, -NHCH3, -NHC(CH3)2, -N(CH3)2, -N(CH2CH3)2, and -NHPh. Examples of cyclic amino groups include, but are not limited to, aziridino, azetidino, pyrrolidino, piperidino, piperazino, morpholino, and thiomorpholino.

Amido (carbamoyl, carbamyl, aminocarbonyl, carboxamide): $-C(=O)NR^1R^2$, wherein R^1 and R^2 are independently amino substituents, as defined for amino groups. Examples of amido groups include, but are not limited to, $-C(=O)NH_2$, $-C(=O)NHCH_3$, $-C(=O)N(CH_3)_2$, $-C(=O)NHCH_2CH_3$, and $-C(=O)N(CH_2CH_3)_2$, as well as amido groups in which R^1 and R^2, together with the nitrogen atom to which they are attached, form a heterocyclic structure as in, for example, piperidinocarbonyl, morpholinocarbonyl, thiomorpholinocarbonyl, and piperazinocarbonyl.

Nitro: $-NO_2$.

Cyano (nitrile, carbonitrile): -CN.

[0141] The term "C_{3-12} alkylene", as used herein, pertains to a bidentate moiety obtained by removing two hydrogen atoms, either both from the same carbon atom, or one from each of

two different carbon atoms, of a hydrocarbon compound having from 3 to 12 carbon atoms (unless otherwise specified), which may be aliphatic or alicyclic, and which may be saturated, partially unsaturated, or fully unsaturated. Thus, the term "alkylene" includes the sub-classes alkenylene, alkynylene, cycloalkylene, etc., discussed below.

[0142] Examples of linear saturated C_{3-12} alkylene groups include, but are not limited to, $-(CH_2)_n-$ where n is an integer from 3 to 12, for example, $-CH_2CH_2CH_2-$ (propylene), $-CH_2CH_2CH_2CH_2-$ (butylene), $-CH_2CH_2CH_2CH_2CH_2-$ (pentylene) and $-CH_2CH_2CH_2CH-_2CH_2CH_2CH_2-$ (heptylene).

[0143] Examples of branched saturated C_{3-12} alkylene groups include, but are not limited to, $-CH(CH_3)CH_2-$, $-CH(CH_3)CH_2CH_2-$, $-CH(CH_3)CH_2CH_2CH_2-$, $-CH_2CH(CH_3)CH_2-$, $-CH_2CH(CH_3)CH_2CH_2-$, $-CH(CH_2CH_3)-$, $-CH(CH_2CH_3)CH_2-$, and $-CH_2CH(CH_2CH_3)CH_2-$.

[0144] Examples of linear partially unsaturated C_{3-12} alkylene groups (C_{3-12} alkenylene, and alkynylene groups) include, but are not limited to, $-CH=CH-CH_2-$, $-CH_2-CH=CH_2-$, $-CH=CH-CH_2-CH_2-$, $-CH=CH-CH_2-CH_2-CH_2-$, $-CH=CH-CH=CH-$, $-CH=CH-CH=CH-CH_2-$, $-CH=CH-CH=CH-CH_2-CH_2-$, $-CH=CH-CH_2-CH=CH-$, $-CH=CH-CH_2-CH_2-CH=CH-$, and $-CH_2-C\equiv C-CH_2-$.

[0145] Examples of branched partially unsaturated C_{3-12} alkylene groups (C_{3-12} alkenylene and alkynylene groups) include, but are not limited to, $-C(CH_3)=CH-$, $-C(CH_3)=CH-CH_2-$, $-CH=CH-CH(CH_3)-$ and $-C\equiv C-CH(CH_3)-$.

[0146] Examples of alicyclic saturated C_{3-12} alkylene groups (C_{3-12} cycloalkylenes) include, but are not limited to, cyclopentylene (e.g. cyclopent-1,3-ylene), and cyclohexylene (e.g. cyclohex-1,4-ylene).

[0147] Examples of alicyclic partially unsaturated C_{3-12} alkylene groups (C_{3-12} cycloalkylenes) include, but are not limited to, cyclopentenylene (e.g. 4-cyclopenten-1,3-ylene), cyclohexenylene (e.g. 2-cyclohexen-1,4-ylene; 3-cyclohexen-1,2-ylene; 2,5-cyclohexadien-1,4-ylene).

Anti-5T4 binding Molecules

[0148] The present disclosure provides anti-5T4 binding molecules, e.g., anti-5T4 antibodies or molecules comprising 5T4-binding fragments thereof, that specifically bind 5T4.

[0149] The full-length amino acid (aa) and nucleotide (nt) sequences for 5T4 are known in the art (see, e.g., UniProt Acc. No. Q13641 for human 5T4).

[0150] In one embodiment, the anti-5T4 binding molecules are antibodies or antigen-binding fragments thereof. In some aspects, the anti-5T4 binding molecules, e.g., anti-5T4 antibodies or molecules comprising anti-5T4-binding fragments thereof, comprise a Fab, a Fab', a F(ab')2, a Fd, a single chain Fv, scFv, disulfide stabilized scFv, a disulfide linked Fv, a V-NAR domain, an IgNar, an intrabody, an IgGΔCH2, a minibody, a F(ab')3, a tetrabody, a triabody, a diabody, a single-domain antibody, DVD-Ig, Fcab, mAb2, a (scFv)2, or a scFv-Fc. In some aspects, the antibody is of the IgG type, for example of the IgG1 type.

CDR Sequences

[0151] In an embodiment an isolated synthetic or recombinant antibody or an antigen binding fragment thereof capable of specifically binding 5T4 cell surface antigen comprises:

a. a heavy chain variable region CDR1 sequence comprising a sequence $GX_1X_2X_3X_4X_5X_6DX_7X_8X_9$, wherein
X_1=F, D or E,
X_2=T, W or G,
X_3=F, A S or T,

X_4= S, A, Q, H, T, G or K,
X_5=N, T, Q, R, A, L, P or S,
X_6=Q, Y, A, L, F, M or S,
X_7=P, W, Q, or L,
X_8=M, L, I, G or V,
X_9=H, S or T,

b. a heavy chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6DX_7X_8X_9X_{10}X_{11}$SAAPVKG, wherein
X_1=R, A, or Q,
X_2=I, V, L, M, or Q,
X_3=R or K,
X_4=A, S, or G,
X_5=Q, K, E, S, V, I, H, L, R, or Y,
X_6=A, Q, R, L, M,
X_7=G, N, E, L,
X_8=G, A, D, E, Q,
X_9=T, E, Q, R, V, W,
X_{10}=T, Q, V, I, P, and
X_{11}=D, Q, V, F, W;

c. a heavy chain variable region CDR3 sequence comprises a sequence that differs by no more than one or two amino acid from the sequence VDRRNYYGMDV (SEQ ID NO: 5;

d. a light chain variable region CDR1 sequence comprises a sequence that differs by no more than one or two amino acids from the sequence RASQGIRNDLG (SEQ ID NO: 12);

e. a light chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6$S, wherein
X_1=A or N,
X_2=A, G, S, T, or V,
X_3=S, W, D, G, N, Q, H, or T,
X_4=R, Q, T, S, H, or K,
X_5=R, L, P, W, H, K, M, N, Q, S, T, or V, and
X_6=D, M, S, or V; and

f. a light chain variable region CDR3 sequence comprising a sequence $X_1QX_2X_3X_4X_5PWX_6$, wherein
X_1=L or V,
X_2=Q, M, or L,
X_3=R, K, Y, F, N, A, H, T, or W,
X_4=R, K, A, N, Q, L, M, S, or T,
X_5=M, T, V, A, I, Q, Y, L, P, K, R, or S, and
X_6=T, R, M, V, A, K, or S.

In an embodiment the antibody, or antigen binding fragment thereof comprises:

a. a heavy chain variable region CDR1 sequence comprising a sequence $GX_1X_2X_3X_4X_5X_6DX_7X_8X_9$, wherein

b. X_1=F, D or E,
X_2=T, W or G,
X_3=F, A S or T,
X_4= S, Q, H, T, or K,
X_5=N, T, L or S,
X_6= Q, Y, A, L, or M,
X_7=P, W, or L,
X_8=M, L, or I
X_9=H, or S;

c. the heavy chain variable region CDR2 sequence comprising a sequence $X_1IX_2AX_3X_4DGGTTDSAAPVKG$, wherein
X_1=R or Q,
X_2=R or K,
X_3=Q, S, V, and
X_4=R, Q, A;

d. a heavy chain variable region CDR3 sequence comprises a sequence that differs by no more than one or two amino acid from the sequence VDRRNYYGMDV (SEQ ID NO: 5);

e. a light chain variable region CDR1 sequence comprises a sequence that differs by no more than one or two amino acids from the sequence RASQGIRNDLG (SEQ ID NO: 12);

f. the light chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6S$, wherein
X_1=A or N,
X_2=G, A, or S,
X_3=W, S, or G,
X_4=Q, T, or R,
X_5=R, L, or P, and
X_6=D, M, S, or V; and

g. the light chain variable region CDR3 sequence comprising a sequence $LQX_1X_2X_3X_4PWX_5$, wherein
X_1=Q, M, L,
X_2=R, Y, K, F, H, N,
X_3=R, K, N, A,
X_4=M, T, V, I, P, and
X_5=T, M, V.

[0152] The VH-CDR3 was not targeted during lead optimisation, as antibodies derived from mouse immunisation hybridomas are often optimised in this region already through somatic hypermutation in vivo and generally targeting the VH-CDR3 does not result in antibodies with large improvements in binding affinity. However, one skilled in the art will appreciate that one or more amino acid changes made within the VH-CDR3 may give rise to variant antibodies with the same or similar binding affinity and specificity.

[0153] It is of note that for the VL-CDR1, targeting this CDR during lead optimisation resulted in no significant improvements in potency versus the parent in the epitope competition assay. However, despite there being no significant improvements over parent, numerous amino acid changes within these regions resulted in antibodies that retained binding and specificity to 5T4, albeit with similar affinity to that of the parental antibody . As such the person skilled in the art will appreciate that a significant degree of variation can be tolerated within VL-CDR1 without abalting the functional characteristics of the antibodies of the present invention.

[0154] In another embodiment, the antibody or antigen binding fragment thereof comprises:

a. a heavy chain variable region CDR1 sequence comprising a sequence that differs by no more than one amino acid from the sequence NAWMS (SEQ ID NO: 3) , SQWMS (SEQ ID NO: 39), or TYPMH (SEQ ID NO: 129);

b. a heavy chain variable region CDR2 sequence comprising a sequence that differs by no more than one or two amino acids from the sequence RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4) or RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58);

c. a heavy chain variable region CDR3 sequence comprising a sequence that differs by no more than one or two amino acid from the sequence VDRRNYYGMDV (SEQ ID NO: 5);

d. a light chain variable region CDR1 sequence comprising a sequence that differs by no more than one or two amino acids from the sequence RASQGIRNDLG (SEQ ID NO: 12);

e. a light chain variable region CDR2 sequence comprising a sequence that differs by no more than one amino acid from the sequence AASSLQS (SEQ ID NO: 13) or AGWQRDS (SEQ ID NO: 85); and

f. a light chain variable region CDR3 sequence comprising a sequence that differs by no more than one amino acid from the sequence LQQNSYPWT (SEQ ID NO: 14), LQQRRMPWT (SEQ ID NO: 122), LQQYRVPWT (SEQ ID NO: 50), or LQMRRTPWT (SEQ ID NO: 104).

[0155] In one embodiment, heavy chain variable region CDR1 comprises NAWMS (SEQ ID NO: 3), SQWMS (SEQ ID NO: 39), or TYPMH (SEQ ID NO: 129).

[0156] In another embodiment, heavy chain variable region CDR2 comprises RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4) or RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58).

[0157] In another embodiment, heavy chain variable region CDR3 comprises VDRRNYYGMDV (SEQ ID NO: 5).

[0158] In a further embodiment, light chain variable region CDR1 comprises RASQGIRNDLG (SEQ ID NO: 12).

[0159] In an additional embodiment, light chain variable region CDR2 comprises AASSLQS (SEQ ID NO: 13) or AGWQRDS (SEQ ID NO: 85);

[0160] In a further embodiment, light chain variable region CDR3 comprises LQQNSYPWT (SEQ ID NO: 14), LQQRRMPWT (SEQ ID NO: 122), LQQYRVPWT (SEQ ID NO: 50), or LQMRRTPWT (SEQ ID NO: 104).

[0161] In one embodiment, the antibody or antigen binding fragment thereof comprises:

a. a heavy chain variable region CDR1 sequence comprising NAWMS (SEQ ID NO: 3) ;

b. a heavy chain variable region CDR2 sequence comprising RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4); and

c. a heavy chain variable region CDR3 sequence comprising VDRRNYYGMDV (SEQ ID NO: 5).

[0162] In one embodiment, the antibody or antigen binding fragment thereof comprises:

a. a heavy chain variable region CDR1 sequence comprising SQWMS (SEQ ID NO: 39);

b. a heavy chain variable region CDR2 sequence comprising RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4); and

c. a heavy chain variable region CDR3 sequence comprising VDRRNYYGMDV (SEQ ID NO: 5).

[0163] In a further embodiment, the antibody or antigen binding fragment thereof comprises:

a. a heavy chain variable region CDR1 sequence comprising NAWMS (SEQ ID NO: 3);

b. a heavy chain variable region CDR2 sequence comprising RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58); and

c. a heavy chain variable region CDR3 sequence comprising VDRRNYYGMDV (SEQ ID NO: 5).

[0164] In a further embodiment, the antibody or antigen binding fragment thereof comprises:

a. a heavy chain variable region CDR1 sequence comprising TYPMH (SEQ ID NO: 129);

b. a heavy chain variable region CDR2 sequence comprising RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4); and

c. a heavy chain variable region CDR3 sequence comprising VDRRNYYGMDV (SEQ ID NO: 5).

[0165] In another embodiment, the antibody or antigen binding fragment thereof comprises:

a. a light chain variable region CDR1 sequence comprising RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprising AASSLQS (SEQ ID NO: 13); and

c. a light chain variable region CDR3 sequence comprising LQQNSYPWT (SEQ ID NO: 14).

[0166] In yet a further embodiment, the antibody or antigen binding fragment thereof comprises:

a. a light chain variable region CDR1 sequence comprises RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprises AASSLQS (SEQ ID NO: 13) or AGWQRDS (SEQ ID NO: 85); and

c. a light chain variable region CDR3 sequence LQQYRVPWT (SEQ ID NO: 50).

[0167] In an additional embodiment, the antibody or antigen binding fragment thereof comprises:

a. a light chain variable region CDR1 sequence comprising RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprising AGWQRDS (SEQ ID NO: 85); and

c. a light chain variable region CDR3 sequence comprising LQQYRVPWT (SEQ ID NO: 50).

[0168] In a further embodiment, the antibody or antigen binding fragment thereof comprises:

a. a light chain variable region CDR1 sequence comprising RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprising AGWQRDS (SEQ ID NO: 85); and

c. a light chain variable region CDR3 sequence comprising LQMRRTPWT (SEQ ID NO: 104).

[0169] In an additional embodiment, the antibody or antigen binding fragment thereof comprises

a. a light chain variable region CDR1 sequence comprising RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprising AASSLQS (SEQ ID NO: 13); and

c. a light chain variable region CDR3 sequence comprising LQQRRMPWT (SEQ ID NO: 122).

[0170] In an additional embodiment, the antibody or antigen binding fragment thereof comprise:

a. a heavy chain variable region CDR1 sequence comprising NAWMS (SEQ ID NO: 3) ;

b. a heavy chain variable region CDR2 sequence comprising RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58);

c. a heavy chain variable region CDR3 sequence comprising VDRRNYYGMDV (SEQ ID NO: 5).

d. a light chain variable region CDR1 sequence comprising RASQGIRNDLG (SEQ ID NO: 12);

e. a light chain variable region CDR2 sequence comprising AGWQRDS (SEQ ID NO: 85); and

f. a light chain variable region CDR3 sequence comprising LQQYRVPWT (SEQ ID NO: 50).

Framework Sequences

[0171] A variety of framework sequences may be used as embodiments, including but not limited to those described in this section.

[0172] In one embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain variable region FW1 sequence comprising EVQLVESGGGLVKPGGSLRLSCAASGX$_1$X$_2$X$_3$X$_4$ (SEQ ID NO: 145),

wherein X_1, X_2, X_3, and X_4 are independently chosen from any amino acid. In one embodiment, X_1 is F or E. In another embodiment, X_2 is T or W. In a further embodiment, X_3 is F or A. In a further embodiment, wherein X_4 is S or Q.

[0173] In a further embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain variable region FW2 sequence comprising WVRQAPGKGLEWIG (SEQ ID NO: 7).

[0174] In a further embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain variable region FW3 sequence comprising RFTISRDDSKNTLYLQMNSLKTEDTAVYYCTT (SEQ ID NO: 8).

[0175] In a further embodiment, the antibody or antigen binding fragment thereof comprises a heavy chain variable region FW4 sequence comprising WGQGTTVTVSS (SEQ ID NO: 9).

[0176] In a further embodiment, the antibody or antigen binding fragment thereof comprises a light chain variable region FW1 sequence comprising DIQMTQSPSSLSASVGDRVTITC (SEQ ID NO: 15).

[0177] In a further embodiment, the antibody or antigen binding fragment thereof comprises a light chain variable region FW2 sequence comprising WYQQKPGKAPKRLIY (SEQ ID NO: 16).

[0178] In an additional embodiment, the antibody or antigen binding fragment thereof comprises a light chain variable region FW3 sequence comprising GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC (SEQ ID NO: 17).

[0179] In an additional embodiment, the antibody or antigen binding fragment thereof comprises a light chain variable region FW4 sequence comprising FGQGTKVEIK (SEQ ID NO: 18).

Additional Modifications

[0180] In one embodiment, the anti-5T4 antibody or antigen binding fragment thereof includes CDRs, each one of which may be at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the CDR's of 3, 4, 5, 12, 13, 14, 21, 22, 23, 30, 31, 32, 39, 40, 41, 48,49, 50, 57, 58, 59, 66, 67, 68, 75, 76, 77, 84, 85, 86, 93, 94, 95, 102, 103, 104, 111, 112, 113, 120, 121, 122, 129, 130, 131, 138, 139 or 140.

[0181] In one embodiment, the anti-5T4 antibody or antigen binding fragment thereof includes a heavy chain, wherein the heavy chain is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 2, 20, 38, 56, 74, 92, 110, or 128.

[0182] In another embodiment, the antibody or antigen binding fragment thereof includes a light chain, wherein the light chain is at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 11, 29, 47, 65, 83, 101, 119, or 137.

[0183] In other embodiments, the substititions within the CDRs are conservative amino acid substitutions. Conservative amino acid substitution involves substitution of one amino acid with another with generally similar properties (size, hydrophobicity, etc.) such that the overall functioning is likely not to be seriously affected. The anti-5T4 antibodies or antigen binding fragments thereof disclosed herein may have one, two or more conservative amino acid substitutions within any individual CDR.

[0184]

Constant Region Modifications to Antibodies or Functional Parts

[0185] Fc domain from an IgG1, IgG2, IgG3, or IgG4. In some aspects, the IgG Fc domain is a human or humanized IgG Fc domain. In some aspects, the Fc domain is an IgG1 Fc domain.

[0186] In some aspects, the IgG Fc domain, for example an IgG1 Fc domain, is a native (wild type) domain. In other aspects, the Fc domain is a mutant IgG domain, for example, a mutant IgG1, IgG2, IgG3, or IgG4 domain. In some specific aspects, the mutant Fc domain is a mutant IgG1 Fc domain.

[0187] In some aspects, the mutant IgG domain, for example, a human or humanized IgG1 Fc domain, comprises at least one mutation capable of reducing the ADCC activity of the anti-5T4 antibody. In certain aspects, at least one mutation capable of reducing the ADCC activity of the anti-5T4 bispecific antibody is an amino acid substitution. Numerous mutations capable of reducing the ADCC activity of an antibody are known in the art. For example, see the mutations described in WO2012175751, WO2011149999, WO2011066501, WO2000042072, WO2011120134, which are herein incorporated by reference in their entireties. Antibodies with reduced ADCC effector function also include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Pat. No. 6,737,056). Such Fc mutants also include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including Fc mutant with substitution of residues 265 and 297 to alanine (U.S. Pat. No. 7,332,581). In some aspects, the anti-5T4 antibody comprises at least one amino acid substitution selected from L234F, S239A, S239C, S442C, or any combination thereof

[0188] In some aspects, the mutant IgG1 Fc domain can comprise at least one amino acid modification introducing a derivatizable functional group. Selectively derivatizable groups are well known in the art, such as a amino group, sulfhydryl group, pendant oxyamino, or other

nucleophilic groups. Derivatizable groups can be joined to a polypeptide chain via one or more linkers. Ligands (*e.g.*, therapeutic agents, detectable labels, half-life extending polymers, etct.) can be attached to the derivatizable groups using the appropriate attachment chemistry. This coupling chemistry can include, for example, amide, urea, thiourea, oxime, aminoacetylamide, etc.

[0189] In some aspects, the derivatizable group is the sulfhydryl side chain of a cysteine amino acid. In particular aspects, the substituted amino acid or amino acids occur at accessible sites of the anti-5T4 binding molecule. By substituting those amino acid residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the anti-5T4 binding molecule and can be used to conjugate the anti-5T4 binding molecule to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate, as described further herein. Cysteine engineered antibodies can be generated as described, *e.g.*, in U.S. Pat. No. 7,521,541.

[0190] In some aspects, the antibody, or antigen binding fragment thereof, contains cysteine residues in the constant region serving as the site of conjugation for a chemotherapeutic or imaging agent. These cysteines may be naturally occurring in the constant region or they may be engineered by an addition or substitution mutation. In one embodiment, the antibody or antigen binding fragment thereof comprises a modified Fc region. The Fc region may comprise at least one engineered cysteine amino acid selected from cysteine amino acid substitutions at amino acid positions 239, 248, 254, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 440, 441, 442, 443 and 446, a cysteine amino acid insertion between positions 239 and 240 (also referenced as iCys for “insertion”, e.g. 239iCys), or any combination thereof, wherein the

amino acid position numbering is according to the EU index as set forth in Kabat (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, VA).

[0191] In some aspects, the amino acid or amino acid substitution introducing a derivatizable sulfhydryl group is selected from the group consisting of S239C, S289C, S339C S442C, or any combination thereof.

[0192] In some aspects, the constant region of the the antibody, or antigen binding fragment thereof, comprises 2 or more engineered cysteine amino acids.

[0193] In some aspects, such modified antibodies, or binding fragments thereof have high stability in serum. In some aspects, such modified antibodies, or binding fragments thereof have improved stability as compared to the parent antibodies from which they are deived, e.g. those parent antibodies having wild-type Fc regions.

[0194] In one embodiment, the antibody or antigen binding fragment thereof has an Fc region having an F at position 234 and a C at position 239, wherein the numbering corresponds to the EU index in Kabat. In another embodiment, the antibody or antigen binding fragment thereof has an Fc region having an F at position 234, a C at position 239, and a C at position 442, wherein the numbering corresponds to the EU index in Kabat. In a further embodiment, the antibody or antigen binding fragment thereof has an Fc region having a C at position 289 and a C at position 339, wherein the numbering corresponds to the EU index in Kabat.

[0195] In another embodiment, the antibody or antigen binding fragment thereof has an Fc region having a C inserted between position 239 and 240 , a C at position 442, or both a C inserted between position 239 and 240 and a C at position 442, wherein the numbering corresponds to the EU index in Kabat.

[0196] In another embodiment, the antibody or antigen binding fragment thereof has additional mutations to ablate ADCC Fc function, including but not limited to an F at position 234 and/or an A at position 239, wherein the numbering corresponds to the EU index in Kabat.

[0197] In one embodiment, additional modifications may be made to antibodies or antigen binding fragment thereof s described herein to improve their half-life. In one embodiment, mutations such as deletion, addition, or substitution mutations may be made to the antibodies or antigen binding fragment thereof s to improve their half-life. In one embodiment, the Fc region may be mutated to include one, two, or all three of the following substitutions M252Y, S254T, and T256E, wherein the numbering corresponds to the EU index in Kabat. In one embodiment, the Fc region may be mutated to include all of the following substitutions M252Y, S254T, and T256E, wherein the numbering corresponds to the EU index in Kabat. Dall'Acqua et al., Properties of Human IgG1s Engineered for Enhanced Binding to the Neonatal Fc Receptor (FcRn), J Biol Chem 281(33):23514-23524 (2006).

Further Characteristics of anti-5T4 antibodies and antigen binding fragment thereof

[0198] In certain embodiments, the antibody or antigen binding fragment thereof has an IC50 of 325 nM or less in a competitive binding assay to 5T4 antigen. In another embodiment, the antibody or antigen binding fragment thereof has an IC50 of 100 nM, 80 nM, 60 nM, 40 nM, 20 nM, 10 nM, 5 nM, or 1 nM or less in a competitive binding assay to 5T4 antigen.

[0199] As used herein, the term antibody or antigen binding fragment thereof is used in the broadest sense. It may be man-made such as monoclonal antibodies (mAbs) produced by conventional hybridoma technology, recombinant technology and/or a functional fragment thereof. It may include both intact immunoglobulin molecules for example a polyclonal antibody, a monoclonal antibody (mAb), a monospecific antibody, a bispecific antibody, a

polyspecific antibody, a fully human antibody, a humanized antibody, an animal antibody (e.g. camelid antibody), chimeric antibodies, as well as portions, fragments, regions, peptides and derivatives thereof (provided by any known technique, such as, but not limited to, enzymatic cleavage, peptide synthesis, or recombinant techniques), such as, for example, immunoglobulin devoid of light chains, Fab, Fab', F $(ab')_2$, Fv, scFv, antibody fragment, diabody, Fd, CDR regions, or any portion or peptide sequence of the antibody that is capable of binding antigen or epitope. In one embodiment, the antigen binding fragment thereof is a single chain antibody, a single chain variable fragment (scFv), a Fab fragment, or a $F(ab')_2$ fragment.

[0200] An antibody or antigen binding fragment thereof is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. Antibody fragments or portions may lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody. Examples of antibody may be produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). Portions of antibodies may be made by any of the above methods, or may be made by expressing a portion of the recombinant molecule. For example, the CDR region(s) of a recombinant antibody may be isolated and subcloned into an appropriate expression vector.

[0201] In one embodiment, an antibody or antigen binding fragment thereof is a human antibody. The use of human antibodies for human therapy may diminish the chance of side effects due to an immunological reaction in a human individual against nonhuman sequences. In another embodiment, the antibody or antigen binding fragment thereof is humanized. In another embodiment, an antibody or antigen binding fragment thereof is a chimeric antibody. This way,

sequences of interest, such as for instance a binding site of interest, can be included into an antibody or antigen binding fragment thereof .

[0202] In one embodiment, the antibody may have an IgG, IgA, IgM, or IgE isotype. In one embodiment, the antibody is an IgG. The antibody may be and IgG1.

Nucleic Acids Encoding Antibodies and Functional Parts Thereof

[0203] The present embodiments further provide an isolated synthetic or recombinant nucleic acid sequence encoding any of the antibodies or antigen binding fragment thereof s described herein. Such nucleic acid is for instance isolated from a B-cell which is capable of producing an antibody or antigen binding fragment thereof . Such nucleic acids encode the heavy and light chain sequences set forth herein. Alternatively, such nucleic acids encode heavy and light chain sequences comprising the heavy and light chain CDRs, respectively, set forth herein. In some embodiments, the nucleic acids will encode antigen binding fragment thereof s of the antibodies described herein. Due to the degeneracy of the nucleic acid code, multiple nucleic acids will encode the same amino acid and all are encompassed herein.

[0204] In one embodiment, a nucleic acid comprises a sequence chosen from sequences encoding the heavy chain: SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127. In another embodiment, a nucleic acid encoding the heavy chain comprises a sequence chosen from one that is 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127. In another embodiment, a nucleic acid sequence comprises sequences encoding heavy chain CDR1, CDR2, and CDR3 wherein the nucleic acids encoding CDR residues are identical to the nucleic acids encoding CDR residues in SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127. In another embodiment, a nucleic acid sequence comprises sequences encoding heavy chain CDR1, CDR2, and CDR3 wherein the nucleic acids encoding

CDR residues result in no more than one or two amino acid differences in each CDR compared to the nucleic acids encoding CDR residues in SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127.

[0205] In one embodiment, a nucleic acid comprises a sequence chosen from sequences encoding the light chain: SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136. In another embodiment, a nucleic acid encoding the light chain comprises a sequence chosen from one that is 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136. In another embodiment, a nucleic acid sequence comprises sequences encoding light chain CDR1, CDR2, and CDR3 wherein the nucleic acids encoding CDR residues are identical to the nucleic acids encoding CDR residues in SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136. In another embodiment, a nucleic acid sequence comprises sequences encoding light chain CDR1, CDR2, and CDR3 wherein the nucleic acids encoding CDR residues result in no more than one or two amino acid differences in each CDR compared to the nucleic acids encoding CDR residues in SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136.

Methods of Making Antibodies and Functional Parts

[0206] An isolated antibody producing cell capable of producing an antibody or antigen binding fragment thereof as disclosed herein is also provided. In some embodiments, antibody producing cells are generated which are stable for at least six months. In another embodiment, an antibody producing cell is stable for at least nine weeks, at least three months, or at least six months. A "stable" cell line means cells that remain capable of producing 5T4-specific antibodies or antigen binding fragment thereof s. In one embodiment, the ability to produce the antibodies or antigen binding fragment thereof s is not decreased; in other embodiments, it decreases only by 5% or less, 10% or less, 15% or less, 20% or less, or 25% or less over the time period in question. In other embodiments, producing any measurable amount of the antibodies or

antigen binding fragment thereof s qualifies a cell line as stable. An antibody producing cell is capable of producing and/or secreting antibody or a functional equivalent thereof, and/or capable of developing into a cell which is capable of producing and/or secreting antibody or a functional equivalent thereof.

[0207] In one embodiment, an antibody producing cell may comprise a mammalian cell. Non-limiting examples include antibody producing cells derived from a human individual, rodent, rabbit, llama, pig, cow, goat, horse, ape, or gorilla. In one embodiment, said antibody producing cell comprises a human cell, a murine cell, a rabbit cell and/or a llama cell.

[0208] In one embodiment, antibody producing cells may comprise Chinese hamster ovary (CHO) cell line, 293(T) cells, COS cells, NS0 cells and other cell lines known in the art and comprise nucleic acid sequences encoding the antibody or antigen binding fragment thereofdescribed herein. Antibody producing cells may be adapted to commercial antibody production ("producer cell"). Proliferation of said producer cell results in a producer cell line capable of producing 5T4-specific antibodies. A producer cell line may be suitable for producing compounds for use in humans. Hence, said producer cell line may be free of pathogenic agents such as pathogenic micro-organisms.

[0209] Further provided is therefore a method for producing antibodies which are capable of specifically binding 5T4, the method comprising: producing an antibody producing cell capable of producing 5T4-specific antibodies and obtaining antibodies produced by said antibody producing cell.

[0210] An isolated or recombinant antibody, as well as an isolated or recombinant antibody producing cell, obtainable by one of the methods embodied herein, or a functional equivalent thereof, is also provided.

[0211] Once a 5T4-specific antibody producing cell is obtained, at least a antigen binding fragment thereof of a gene encoding the Ig heavy chain and/or light chain of said cell may be isolated and/or generated artificially.

Antibody-Drug Conjugates

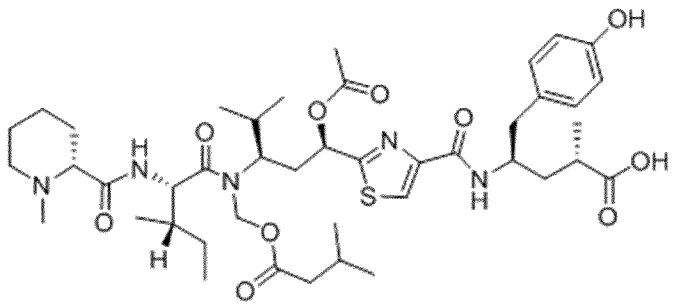
[0212] Antibodies and antigen binding fragments thereof described herein may be conjugated to a drug to form an antibody-drug conjugate. The use of the phrase antibody-drug conjugates specifically contemplates the use of an antigen binding fragment of an antibody as the antibody portion in the conjugate.

[0213] Drugs that may be conjugated to a the antibodies and antigen binding fragments thereof described herein include

[0214] In one embodiment, the antibody-drug conjugate comprises an antibody as described herein and a cytotoxic agent. In one embodiment, the cytotoxic agent is a cancer chemotherapeutic agent. The chemotherapeutic agent may be an amanitin, auristatin, daunomycin, doxorubicin, duocarmycin, dolastatin, enediyne, lexitropsin, taxane, puromycin, maytansinoid, vinca alkaloid, tubulysin or pyrrolobenzodiazepine. In particular embodiments, the cytotoxic agents may be AFP, MMAF, MMAE, AEB, AEVB, auristatin E, paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretatstatin, chalicheamicin, maytansine, DM-1, vinblastine, methotrexate, or netropsin.

Tubulysins

[0215] In one embodiment, the cytotoxic agent is a tubulysin or tubulysin derivative. Tubulysin A has the following chemical structure:

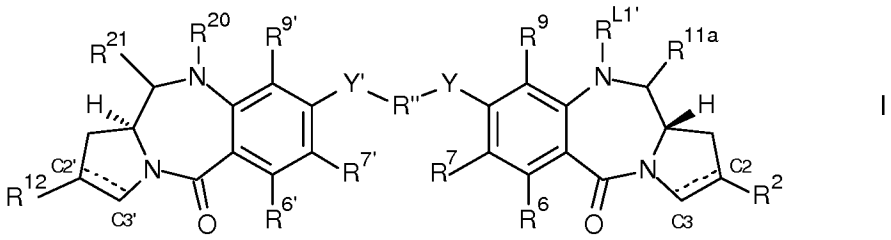


tubulysin A

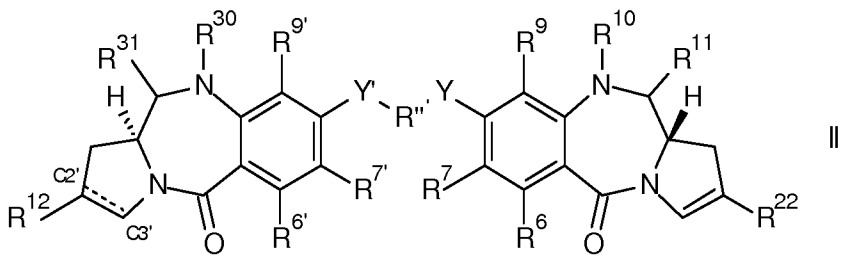
Pyrrolobenzodiazepines

[0216] In another embodiment, the cytotoxic agent may be a pyrrolobenzodiazepine or a pyrrolobenzodiazepine derivative.

[0217] The pyrrolobenzodiazepines or pyrrolobenzodiazepine derivatives (PBDs) may have a labile C2 or N10 protecting group in the form of a linker to an antibody. In general they can be described by the following formulae I and II:



I



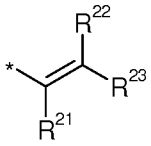
II

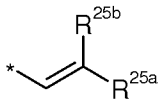
wherein when there is a double bond present between C2' and C3', R12 is selected from the group consisting of:

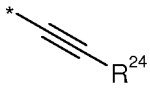
(ia) C5-10 aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C1-7 alkyl, C3-7 heterocyclyl and bis-oxy-C1-3 alkylene;

(ib) C1-5 saturated aliphatic alkyl;

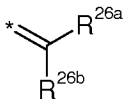
(ic) C3-6 saturated cycloalkyl;

(id)  , wherein each of R21, R22 and R23 are independently selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl, where the total number of carbon atoms in the R12 group is no more than 5;

(ie)  , wherein one of R25a and R25b is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and

(if)  , where R24 is selected from: H; C1-3 saturated alkyl; C2-3 alkenyl; C2-3 alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

when there is a single bond present between C2' and C3',

R12 is  , where R26a and R26b are independently selected from H, F, C1-4 saturated alkyl, C2-3 alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C1-4 alkyl amido and C1-4 alkyl ester; or, when one of R26a and R26b is H, the other is selected from nitrile and a C1-4 alkyl ester;

R6 and R9 are independently selected from H, R, OH, OR, SH, SR, NH2, NHR, NRR', nitro, Me3Sn and halo;

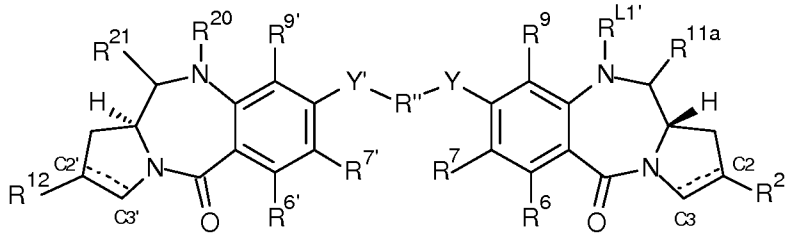
where R and R' are independently selected from optionally substituted C1-12 alkyl, C3-7 heterocyclyl and C5-10 aryl groups;

R7 is selected from H, R, OH, OR, SH, SR, NH2, NHR, NHRR', nitro, Me3Sn and halo;

R″ is a C3-12 alkylene group, which chain may be interrupted by one or more heteroatoms, e.g. O, S, NRN2 (where RN2 is H or C1-4 alkyl), and/or aromatic rings, e.g. benzene or pyridine;

Y and Y' are selected from O, S, or NH;

R6', R7', R9' are selected from the same groups as R6, R7 and R9 respectively;



I

[Formula I]

RL1' is a linker for connection to the antibody;

R11a is selected from OH, ORA, where RA is C1-4 alkyl, and SOzM, where z is 2 or 3 and M is a monovalent pharmaceutically acceptable cation;

R20 and R21 either together form a double bond between the nitrogen and carbon atoms to which they are bound or;

R20 is selected from H and RC, where RC is a capping group;

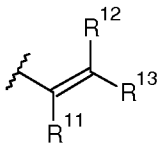
R21 is selected from OH, ORA and SOzM;

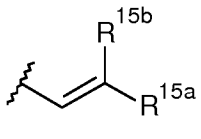
when there is a double bond present between C2 and C3, R2 is selected from the group consisting of:

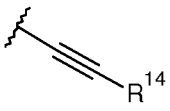
(ia) C5-10 aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C1-7 alkyl, C3-7 heterocyclyl and bis-oxy-C1-3 alkylene;

(ib) C1-5 saturated aliphatic alkyl;

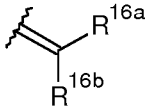
(ic) C3-6 saturated cycloalkyl;

(id)  , wherein each of R11, R12 and R13 are independently selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl, where the total number of carbon atoms in the R2 group is no more than 5;

(ie)  , wherein one of R15a and R15b is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and

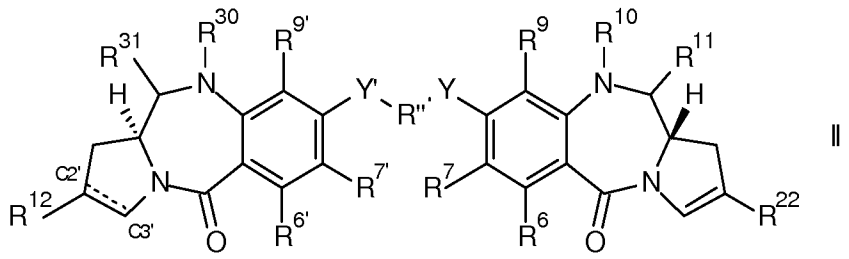
(if)  , where R14 is selected from: H; C1-3 saturated alkyl; C2-3 alkenyl; C2-3 alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

when there is a single bond present between C2 and C3,



R2 is, where R16a and R16b are independently selected from H, F, C1-4 saturated alkyl, C2-3 alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C1-4

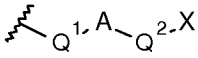
alkyl amido and C1-4 alkyl ester; or, when one of R16a and R16b is H, the other is selected from nitrile and a C1-4 alkyl ester;



II

[Formula II]

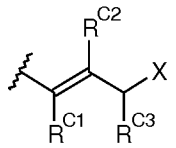
R22 is of formula IIIa, formula IIIb or formula IIIc:

(a)  IIIa

where A is a C5-7 aryl group, and either

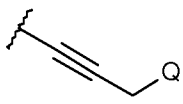
(i) Q1 is a single bond, and Q2 is selected from a single bond and -Z-(CH2)n-, where Z is selected from a single bond, O, S and NH and n is from 1 to 3; or

(ii) Q1 is -CH=CH-, and Q2 is a single bond;

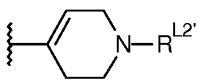
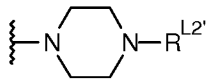
(b)  IIIb

where;

RC1, RC2 and RC3 are independently selected from H and unsubstituted C1-2 alkyl;

(c)  IIIc

where Q is selected from O-RL2', S-RL2' and NRN-RL2', and RN is selected from H, methyl and ethyl

X is selected from the group comprising: O-RL2', S-RL2', CO2-RL2', CO-RL2', NH-C(=O)-RL2', NHNH-RL2', CONHNH-RL2',  ,  , NRNRL2',

wherein RN is selected from the group comprising H and C1-4 alkyl;

RL2' is a linker for connection to the antibody (Ab);

R10 and R11 either together form a double bond between the nitrogen and carbon atoms to which they are bound or;

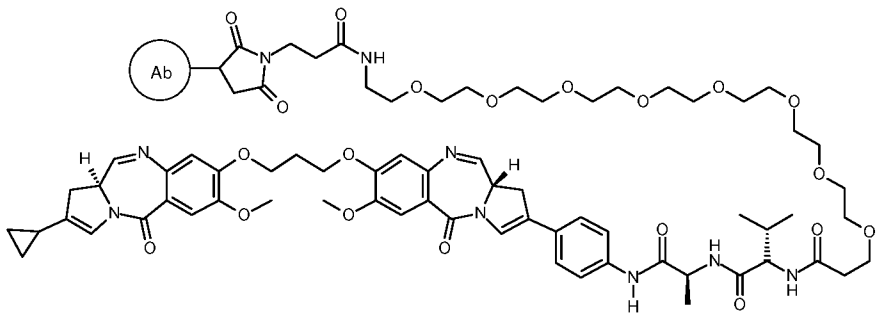
R10 is H and R11 is selected from OH, ORA and SOzM;

R30 and R31 either together form a double bond between the nitrogen and carbon atoms to which they are bound or;

R30 is H and R31 is selected from OH, ORA and SOzM.

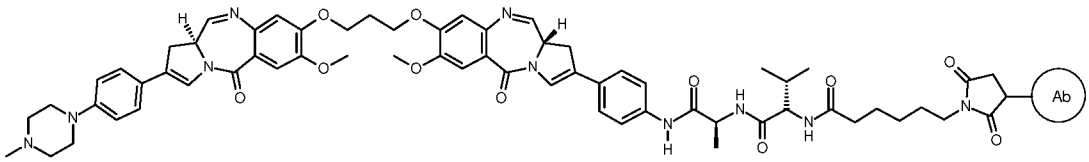
[0218] In some embodiments, it may be preferred that the conjugate is selected from a conjugate of formula ConjA, ConjB, ConjC, ConjD, ConjE or ConjF :

ConjA (made up from Ab & compound 18)



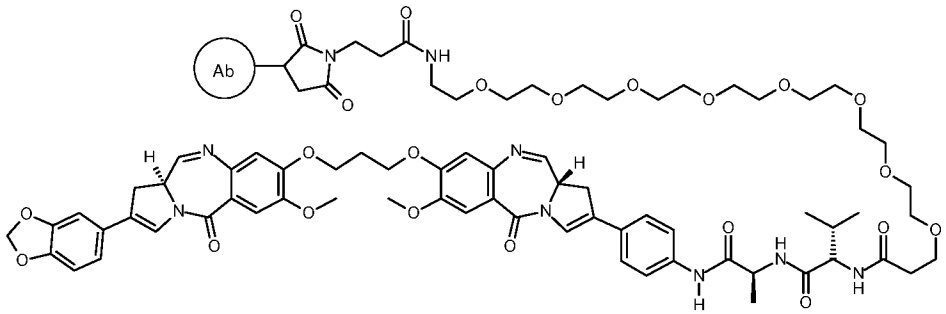
ConjA ;

ConjB (made up from Ab & compound 28)



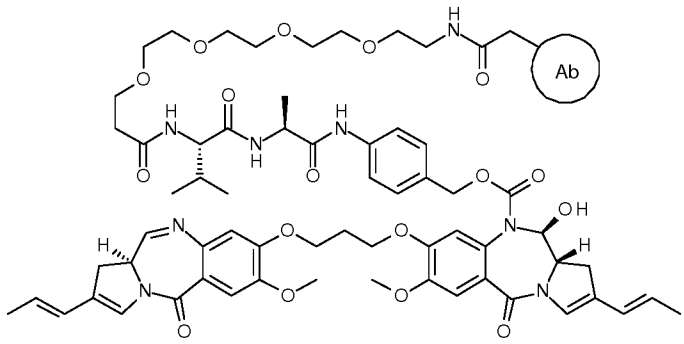
ConjB ;

ConjC(made up from Ab & compound 33):



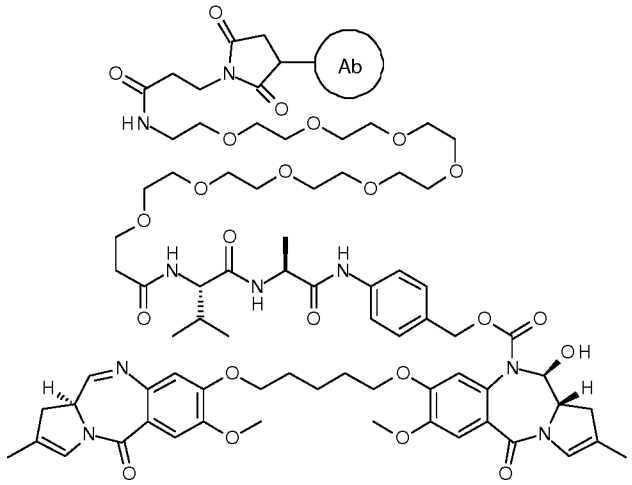
ConjC ;

ConjD(made up from Ab & compound 66)



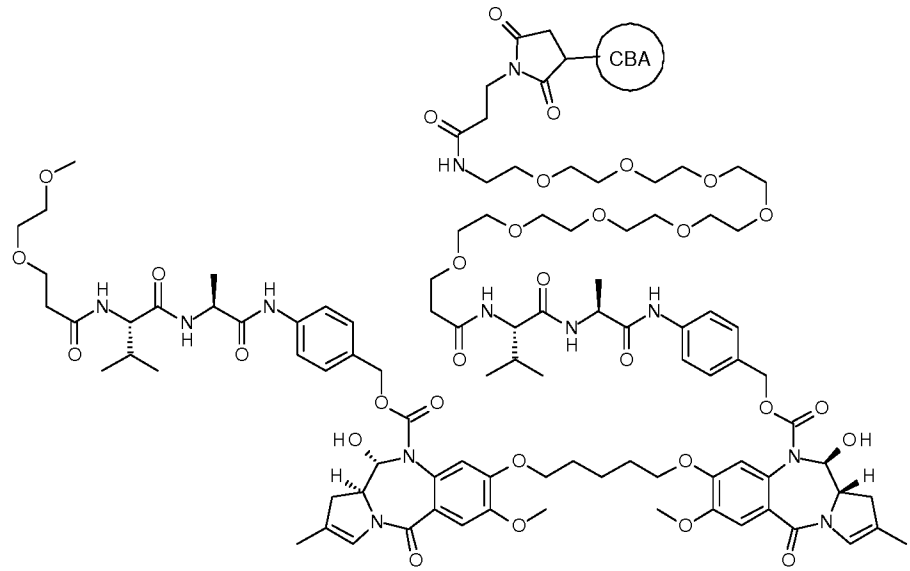
ConjD ;

ConjE(made up from Ab & compound 64):



ConjE ; or

ConjF(made up from Ab & compound 96):

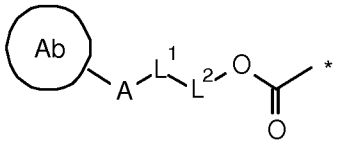


ConjF

PBD Conjugates

[0219] RL' may be either RL1' or RL2'.

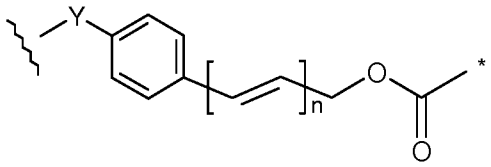
[0220] In one embodiment, Ab-RL' is a group:



where the asterisk indicates the point of attachment to the PBD of formula I or II, Ab is the antibody, L1 is a linker, A is a connecting group connecting L1 to the antibody, L2 is a covalent bond or together with -OC(=O)- forms a self-immolative linker, and L1 or L2 is a cleavable linker. Such groups are described in WO 2011/130598 , WO 2011/130613 , WO 2011/130616 , WO 2013/053873 , WO 2013/053871 and WO 2013/041606 .

[0221] In one embodiment, L2 is present and together with -C(=O)O- forms a self-immolative linker. In one embodiment, L2 is a substrate for enzymatic activity, thereby allowing release of L-RL' from the N10 position.

[0222] In one embodiment, -C(=O)O- and L2 together form the group:



where the asterisk indicates the point of attachment to the N10 position, the wavy line indicates the point of attachment to the linker L1, Y is -N(H)-, -O-, -C(=O)N(H)- or -C(=O)O-, and n is 0 to 3. The phenylene ring is optionally substituted with one, two or three substituents as described herein. In one embodiment, the phenylene group is optionally substituted with halo, NO2, R or OR.

[0223] In one embodiment, Y is NH.

[0224] In one embodiment, n is 0 or 1. Preferably, n is 0.

[0225] Where Y is NH and n is 0, the self-immolative linker may be referred to as a p-aminobenzylcarbonyl linker (PABC).

[0226] In one embodiment, L1 comprises a dipeptide The dipeptide may be represented as -NH-X1-X2-CO-, where -NH- and -CO- represent the N- and C-terminals of the amino acid groups X1 and X2 respectively. The amino acids in the dipeptide may be any combination of natural amino acids. Where the linker is a cathepsin labile linker, the dipeptide may be the site of action for cathepsin-mediated cleavage.

[0227] Additionally, for those amino acids groups having carboxyl or amino side chain functionality, for example Glu and Lys respectively, CO and NH may represent that side chain functionality.

[0228] In one embodiment, the group -X1-X2- in dipeptide, -NH-X1-X2-CO-, is selected from:

-Phe-Lys-,

-Val-Ala-,

-Val-Lys-,

-Ala-Lys-,

-Val-Cit-,

-Phe-Cit-,

-Leu-Cit-,

-Ile-Cit-,

-Phe-Arg-,

-Trp-Cit-

where Cit is citrulline.

[0229] Preferably, the group -X1-X2- in dipeptide, -NH-X1-X2-CO-, is selected from:

-Phe-Lys-,

-Val-Ala-,

-Val-Lys-,

-Ala-Lys-,

-Val-Cit-.

[0230] Most preferably, the group -X1-X2- in dipeptide, -NH-X1-X2-CO-, is -Phe-Lys- or -Val-Ala-.

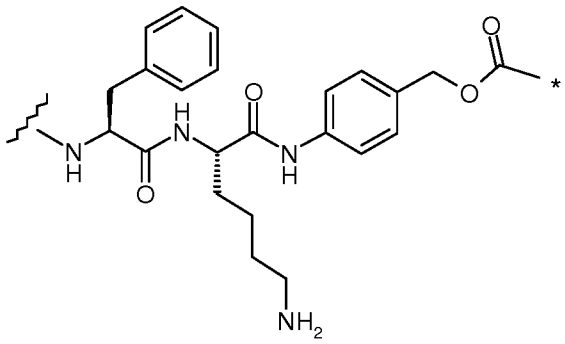
[0231] Other dipeptide combinations may be used, including those described by Dubowchik et al., Bioconjugate Chemistry, 2002, 13,855-869, which is incorporated herein by reference.

[0232] In one embodiment, the dipeptide is used in combination with a self-immolative linker. The self-immolative linker may be connected to -X2-.

[0233] Where a self-immolative linker is present, -X2- is connected directly to the self-immolative linker. Preferably the group -X2-CO- is connected to Y, where Y is NH, thereby forming the group -X2-CO-NH-.

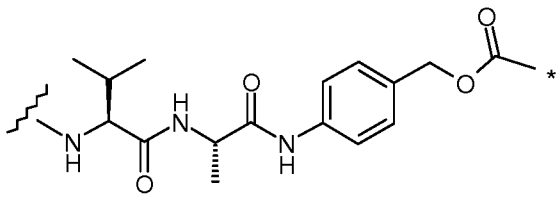
-NH-X1- is connected directly to A. A may comprise the functionality -CO- thereby to form an amide link with -X1-.

[0234] In one embodiment, L1 and L2 together with -OC(=O)- comprise the group NH-X1-X2-CO-PABC-. The PABC group is connected directly to the N10 position. Preferably, the self-immolative linker and the dipeptide together form the group -NH-Phe-Lys-CO-NH-PABC-, which is illustrated below:



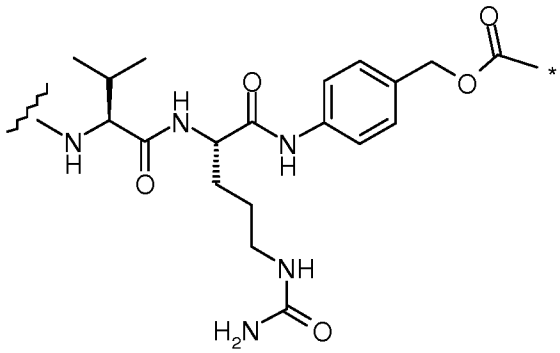
where the asterisk indicates the point of attachment to the N10 position, and the wavy line indicates the point of attachment to the remaining portion of the linker L1 or the point of attachment to A. Preferably, the wavy line indicates the point of attachment to A. The side chain of the Lys amino acid may be protected, for example, with Boc, Fmoc, or Alloc, as described above.

[0235] Alternatively, the self-immolative linker and the dipeptide together form the group -NH-Val-Ala-CO-NH-PABC-, which is illustrated below:



where the asterisk and the wavy line are as defined above.

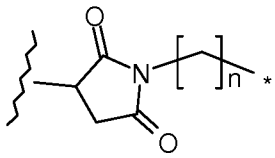
[0236] Alternatively, the self-immolative linker and the dipeptide together form the group -NH-Val-Cit-CO-NH-PABC-, which is illustrated below:



where the asterisk and the wavy line are as defined above.

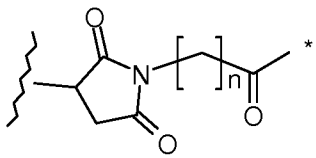
[0237] In one embodiment, A is a covalent bond. Thus, L1 and the antibody are directly connected. For example, where L1 comprises a contiguous amino acid sequence, the N-terminus of the sequence may connect directly to the antibody.

[0238] In one embodiment, the group A is:



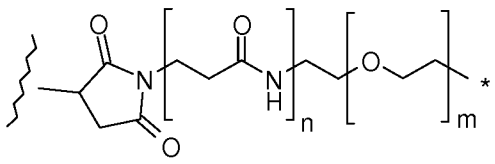
where the asterisk indicates the point of attachment to L1, the wavy line indicates the point of attachment to the antibody, and n is 0 to 6. In one embodiment, n is 5.

[0239] In one embodiment, the group A is:



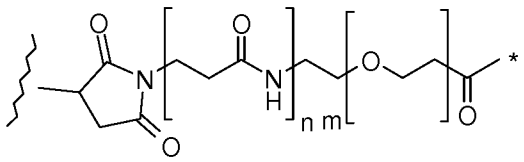
where the asterisk indicates the point of attachment to L1, the wavy line indicates the point of attachment to the antibody, and n is 0 to 6. In one embodiment, n is 5.

[0240] In one embodiment, the group A is:



where the asterisk indicates the point of attachment to L1, the wavy line indicates the point of attachment to the antibody, n is 0 or 1, and m is 0 to 30. In a preferred embodiment, n is 1 and m is 0 to 10, 1 to 8, preferably 4 to 8, and most preferably 4 or 8. In another embodiment, m is 10 to 30, and preferably 20 to 30. Alternatively, m is 0 to 50. In this embodiment, m is preferably 10-40 and n is 1.

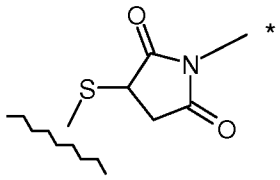
[0241] In one embodiment, the group A is:



where the asterisk indicates the point of attachment to L1, the wavy line indicates the point of attachment to the antibody, n is 0 or 1, and m is 0 to 30. In a preferred embodiment, n is 1 and m is 0 to 10, 1 to 8, preferably 4 to 8, and most preferably 4 or 8. In another embodiment, m is 10 to 30, and preferably 20 to 30. Alternatively, m is 0 to 50. In this embodiment, m is preferably 10-40 and n is 1.

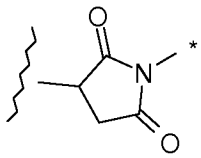
[0242] In one embodiment, the connection between the antibody and A is through a thiol residue of the antibody and a maleimide group of A.

[0243] In one embodiment, the connection between the antibody and A is:



where the asterisk indicates the point of attachment to the remaining portion of A and the wavy line indicates the point of attachment to the remaining portion of the antibody. In this embodiment, the S atom is typically derived from the antibody.

[0244] In each of the embodiments above, an alternative functionality may be used in place of the maleimide-derived group shown below:



[0245] where the wavy line indicates the point of attachment to the antibody as before, and the asterisk indicates the bond to the remaining portion of the A group.

[0246] In one embodiment, the maleimide-derived group is replaced with a group, which optionally together with the antibody, is selected from:

-C(=O)NH-,

-C(=O)O-,

-NHC(=O)-,

-OC(=O)-,

-OC(=O)O-,

-NHC(=O)O-,

-OC(=O)NH-,

-NHC(=O)NH-,

-NHC(=O)NH,

-C(=O)NHC(=O)-,

-S-,

-S-S-,

-CH2C(=O)-

-C(=O)CH2-,

=N-NH-, and

-NH-N=.

[0247] Other groups suitable for connecting L1 to the antibody are described in WO 2005/082023.

Rc, Capping Group

[0248] The conjugate of the first aspect of the invention may have a capping group RC at the N10 position. Such groups are described in WO 2011/130598 on pages 72 to 77.

[0249] Embodiments of the present invention include ConjA wherein the antibody is as defined above.

[0250] Embodiments of the present invention include ConjB wherein the antibody is as defined above.

[0251] Embodiments of the present invention include ConjC wherein the antibody is as defined above.

[0252] Embodiments of the present invention include ConjD wherein the antibody is as defined above.

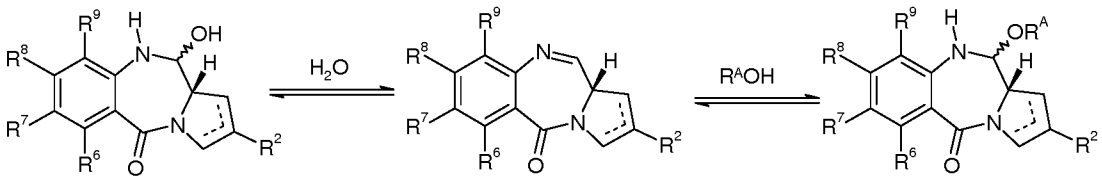
[0253] Embodiments of the present invention include ConjE wherein the antibody is as defined above.

[0254] Embodiments of the present invention include ConjF wherein the antibody is as defined above.

Solvates

[0255] It may be convenient or desirable to prepare, purify, and/or handle a corresponding solvate of the active compound. The term "solvate" is used herein in the conventional sense to refer to a complex of solute (e.g. active compound, salt of active compound) and solvent. If the solvent is water, the solvate may be conveniently referred to as a hydrate, for example, a mono-hydrate, a di-hydrate, a tri-hydrate, etc.

[0256] The invention includes compounds where a solvent adds across the imine bond of the PBD moiety, which is illustrated below where the solvent is water or an alcohol (RAOH, where RA is C1-4 alkyl):



[0257] These forms can be called the carbinolamine and carbinolamine ether forms of the PBD (as described in the section relating to R10 above). The balance of these equilibria depend on the conditions in which the compounds are found, as well as the nature of the moiety itself.

[0258] These particular compounds may be isolated in solid form, for example, by lyophilisation.

[0259] The following preferences may apply to all aspects of the conjugates as described above, or may relate to a single aspect. The preferences may be combined together in any combination.

[0260] In some embodiments, R6', R7', R9', and Y' are preferably the same as R6, R7, R9, and Y respectively.

Dimer link

Y and Y' are preferably O.

R'' is preferably a C3-7 alkylene group with no substituents. More preferably R'' is a C3, C5 or C7 alkylene. Most preferably, R'' is a C3 or C5 alkylene.

R6 to R9

R9 is preferably H.

R6 is preferably selected from H, OH, OR, SH, NH2, nitro and halo, and is more preferably H or halo, and most preferably is H.

[0261] R7 is preferably selected from H, OH, OR, SH, SR, NH2, NHR, NRR', and halo, and more preferably independently selected from H, OH and OR, where R is preferably selected from optionally substituted C1-7 alkyl, C3-10 heterocyclyl and C5-10 aryl groups. R may be more preferably a C1-4 alkyl group, which may or may not be substituted. A substituent of interest is a C5-6 aryl group (e.g. phenyl). Particularly preferred substituents at the 7- positions are OMe and OCH2Ph. Other substituents of particular interest are dimethylamino (i.e. –NMe2); -(OC2H4)qOMe, where q is from 0 to 2; nitrogen-containing C6 heterocyclyls, including morpholino, piperidinyl and N-methyl-piperazinyl.

[0262] These preferences apply to R9', R6' and R7' respectively.

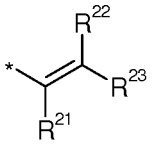
[0263] R12

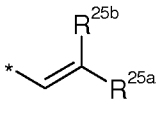
[0264] When there is a double bond present between C2' and C3', R12 is selected from:

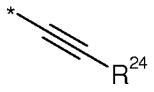
[0265] (a) C5-10 aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, C1-7 alkyl, C3-7 heterocyclyl and bis-oxy-C1-3 alkylene;

[0266] (b) C1-5 saturated aliphatic alkyl;

[0267] (c) C3-6 saturated cycloalkyl;

[0268] (d)  , wherein each of R21, R22 and R23 are independently selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl, where the total number of carbon atoms in the R12 group is no more than 5;

[0269] (e)  , wherein one of R25a and R25b is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl; and

[0270] (f)  , where R24 is selected from: H; C1-3 saturated alkyl; C2-3 alkenyl; C2-3 alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl.

[0271] When R12 is a C5-10 aryl group, it may be a C5-7 aryl group. A C5-7 aryl group may be a phenyl group or a C5-7 heteroaryl group, for example furanyl, thiophenyl and pyridyl. In some embodiments, R12 is preferably phenyl. In other embodiments, R12 is preferably thiophenyl, for example, thiophen-2-yl and thiophen-3-yl.

[0272] When R12 is a C5-10 aryl group, it may be a C8-10 aryl, for example a quinolinyl or isoquinolinyl group. The quinolinyl or isoquinolinyl group may be bound to the PBD core

through any available ring position. For example, the quinolinyl may be quinolin-2-yl, quinolin-3-yl, quinolin-4yl, quinolin-5-yl, quinolin-6-yl, quinolin-7-yl and quinolin-8-yl. Of these quinolin-3-yl and quinolin-6-yl may be preferred. The isoquinolinyl may be isoquinolin-1-yl, isoquinolin-3-yl, isoquinolin-4yl, isoquinolin-5-yl, isoquinolin-6-yl, isoquinolin-7-yl and isoquinolin-8-yl. Of these isoquinolin-3-yl and isoquinolin-6-yl may be preferred.

[0273] When R12 is a C5-10 aryl group, it may bear any number of substituent groups. It preferably bears from 1 to 3 substituent groups, with 1 and 2 being more preferred, and singly substituted groups being most preferred. The substituents may be any position.

[0274] Where R12 is C5-7 aryl group, a single substituent is preferably on a ring atom that is not adjacent the bond to the remainder of the compound, i.e. it is preferably β or γ to the bond to the remainder of the compound. Therefore, where the C5-7 aryl group is phenyl, the substituent is preferably in the meta- or para- positions, and more preferably is in the para-position.

[0275] Where R12 is a C8-10 aryl group, for example quinolinyl or isoquinolinyl, it may bear any number of substituents at any position of the quinoline or isoquinoline rings. In some embodiments, it bears one, two or three substituents, and these may be on either the proximal and distal rings or both (if more than one substituent).

[0276] R12 substituents, when R12 is a C5-10 aryl group

[0277] If a substituent on R12 when R12 is a C5-10 aryl group is halo, it is preferably F or Cl, more preferably Cl.

[0278] If a substituent on R12 when R12 is a C5-10 aryl group is ether, it may in some embodiments be an alkoxy group, for example, a C1-7 alkoxy group (e.g. methoxy, ethoxy) or it may in some embodiments be a C5-7 aryloxy group (e.g phenoxy, pyridyloxy, furanyloxy). The

alkoxy group may itself be further substituted, for example by an amino group (e.g. dimethylamino).

If a substituent on R12 when R12 is a C5-10 aryl group is C1-7 alkyl, it may preferably be a C1-4 alkyl group (e.g. methyl, ethyl, propryl, butyl).

[0279] If a substituent on R12 when R12 is a C5-10 aryl group is C3-7 heterocyclyl, it may in some embodiments be C6 nitrogen containing heterocyclyl group, e.g. morpholino, thiomorpholino, piperidinyl, piperazinyl. These groups may be bound to the rest of the PBD moiety via the nitrogen atom. These groups may be further substituted, for example, by C1-4 alkyl groups. If the C6 nitrogen containing heterocyclyl group is piperazinyl, the said further substituent may be on the second nitrogen ring atom.

[0280] If a substituent on R12 when R12 is a C5-10 aryl group is bis-oxy-C1-3 alkylene, this is preferably bis-oxy-methylene or bis-oxy-ethylene.

[0281] If a substituent on R12 when R12 is a C5-10 aryl group is ester, this is preferably methyl ester or ethyl ester.

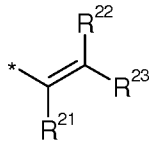
[0282] Particularly preferred substituents when R12 is a C5-10 aryl group include methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl. Other particularly preferred substituent for R12 are dimethylaminopropyloxy and carboxy.

[0283] Particularly preferred substituted R12 groups when R12 is a C5-10 aryl group include, but are not limited to, 4-methoxy-phenyl, 3-methoxyphenyl, 4-ethoxy-phenyl, 3-ethoxy-phenyl, 4-fluoro-phenyl, 4-chloro-phenyl, 3,4-bisoxymethylene-phenyl, 4-methylthiophenyl, 4-cyanophenyl, 4-phenoxyphenyl, quinolin-3-yl and quinolin-6-yl, isoquinolin-3-yl and isoquinolin-6-yl, 2-thienyl, 2-furanyl, methoxynaphthyl, and naphthyl. Another possible

substituted R12 group is 4-nitrophenyl. R12 groups of particular interest include 4-(4-methylpiperazin-1-yl)phenyl and 3,4-bisoxymethylene-phenyl.

[0284] When R12 is C1-5 saturated aliphatic alkyl, it may be methyl, ethyl, propyl, butyl or pentyl. In some embodiments, it may be methyl, ethyl or propyl (n-pentyl or isopropyl). In some of these embodiments, it may be methyl. In other embodiments, it may be butyl or pentyl, which may be linear or branched.

[0285] When R12 is C3-6 saturated cycloalkyl, it may be cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl. In some embodiments, it may be cyclopropyl.

[0286] When R12 is  , each of R21, R22 and R23 are independently selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl, where the total number of carbon atoms in the R12 group is no more than 5. In some embodiments, the total number of carbon atoms in the R12 group is no more than 4 or no more than 3.

[0287] In some embodiments, one of R21, R22 and R23 is H, with the other two groups being selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl.

[0288] In other embodiments, two of R21, R22 and R23 are H, with the other group being selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl.

[0289] In some embodiments, the groups that are not H are selected from methyl and ethyl. In some of these embodiments, the groups that re not H are methyl.

[0290] In some embodiments, R21 is H.

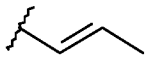
[0291] In some embodiments, R22 is H.

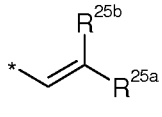
[0292] In some embodiments, R23 is H.

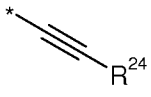
[0293] In some embodiments, R21 and R22 are H.

[0294] In some embodiments, R21 and R23 are H.

[0295] In some embodiments, R22 and R23 are H.

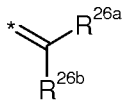
[0296] An R12 group of particular interest is:  .

[0297] When R12 is  , one of R25a and R25b is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl. In some embodiments, the group which is not H is optionally substituted phenyl. If the phenyl optional substituent is halo, it is preferably fluoro. In some embodiment, the phenyl group is unsubstituted.

[0298] When R12 is  , R24 is selected from: H; C1-3 saturated alkyl; C2-3 alkenyl; C2-3 alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo methyl, methoxy; pyridyl; and thiophenyl. If the phenyl optional substituent is halo, it is preferably fluoro. In some embodiment, the phenyl group is unsubstituted.

[0299] In some embodiments, R24 is selected from H, methyl, ethyl, ethenyl and ethynyl. In some of these embodiments, R24 is selected from H and methyl.

[0300] When there is a single bond present between C2' and C3',

[0301] R12 is  , where R26a and R26b are independently selected from H, F, C1-4 saturated alkyl, C2-3 alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C1-4 alkyl amido and C1-4 alkyl ester; or, when one of R26a and R26b is H, the other is selected from nitrile and a C1-4 alkyl ester.

[0302] In some embodiments, it is preferred that R26a and R26b are both H.

[0303] In other embodiments, it is preferred that R26a and R26b are both methyl.

[0304] In further embodiments, it is preferred that one of R26a and R26b is H, and the other is selected from C1-4 saturated alkyl, C2-3 alkenyl, which alkyl and alkenyl groups are optionally substituted. In these further embodiment, it may be further preferred that the group which is not H is selected from methyl and ethyl.

[0305] R2

The above preferences for R12 apply equally to R2.

[0306] R22

[0307] In some embodiments, R22 is of formula IIa.

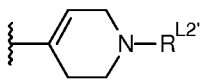
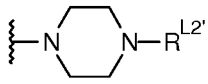
[0308] A in R22 when it is of formula IIa may be phenyl group or a C5-7 heteroaryl group, for example furanyl, thiophenyl and pyridyl. In some embodiments, A is preferably phenyl.

[0309] Q2-X may be on any of the available ring atoms of the C5-7 aryl group, but is preferably on a ring atom that is not adjacent the bond to the remainder of the compound, i.e. it is preferably β or γ to the bond to the remainder of the compound. Therefore, where the C5-7 aryl group (A) is phenyl, the substituent (Q2-X) is preferably in the meta- or para- positions, and more preferably is in the para- position.

[0310] In some embodiments, Q1 is a single bond. In these embodiments, Q2 is selected from a single bond and -Z-(CH2)n-, where Z is selected from a single bond, O, S and NH and is from 1 to 3. In some of these embodiments, Q2 is a single bond. In other embodiments, Q2 is -Z-(CH2)n-. In these embodiments, Z may be O or S and n may be 1 or n may be 2. In other of these embodiments, Z may be a single bond and n may be 1.

[0311] In other embodiments, Q1 is -CH=CH-.

[0312] In other embodiments, R22 is of formula IIb. In these embodiments, RC1, RC2 and RC3 are independently selected from H and unsubstituted C1-2 alkyl. In some preferred embodiments, RC1, RC2 and RC3 are all H. In other embodiments, RC1, RC2 and RC3 are all methyl. In certain embodiments, RC1, RC2 and RC3 are independently selected from H and methyl.

[0313] X is a group selected from the list comprising: O-RL2', S-RL2', CO2-RL2', CO-RL2', NH-C(=O)-RL2', NHNH-RL2', CONHNH-RL2',  ,  , NRNRL2', wherein RN is selected from the group comprising H and C1-4 alkyl. X may preferably be: OH, SH, CO2H, -N=C=O or NHRN, and may more preferably be: O-RL2', S-RL2', CO2-RL2', -NH-C(=O)-RL2' or NH-RL2'. Particularly preferred groups include: O-RL2', S-RL2' and NH-RL2', with NH-RL2' being the most preferred group.

[0314] In some embodiments R22 is of formula IIc. In these embodiments, it is preferred that Q is NRN-RL2'. In other embodiments, Q is O-RL2'. In further embodiments, Q is S-RL2'. RN is preferably selected from H and methyl. In some embodiment, RN is H. In other embodiments, RN is methyl.

[0315] In some embodiments, R22 may be -A-CH2-X and -A-X. In these embodiments, X may be O-RL2', S-RL2', CO2-RL2', CO-RL2' and NH-RL2'. In particularly preferred embodiments, X may be NH-RL2'.

[0316] R10, R11

[0317] In some embodiments, R10 and R11 together form a double bond between the nitrogen and carbon atoms to which they are bound.

[0318] In some embodiments, R11 is OH.

[0319] In some embodiments, R11 is OMe.

[0320] In some embodiments, R11 is SOzM, where z is 2 or 3 and M is a monovalent pharmaceutically acceptable cation.

[0321] R11a

[0322] In some embodiments, R11a is OH.

[0323] In some embodiments, R11a is OMe.

[0324] In some embodiments, R11a is SOzM, where z is 2 or 3 and M is a monovalent pharmaceutically acceptable cation.

[0325] R20, R21

[0326] In some embodiments, R20 and R21 together form a double bond between the nitrogen and carbon atoms to which they are bound.

[0327] In some embodiments R20 is H.

[0328] In some embodiments, R20 is RC.

[0329] In some embodiments, R21 is OH.

[0330] In some embodiments, R21 is OMe.

[0331] In some embodiments, R21 is SOzM, where z is 2 or 3 and M is a monovalent pharmaceutically acceptable cation.

[0332] R30, R31

[0333] In some embodiments, R30 and R31 together form a double bond between the nitrogen and carbon atoms to which they are bound.

[0334] In some embodiments, R31 is OH.

[0335] In some embodiments, R31 is OMe.

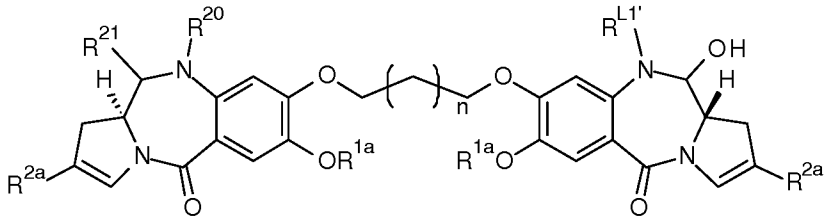
[0336] In some embodiments, R31 is SOzM, where z is 2 or 3 and M is a monovalent pharmaceutically acceptable cation.

[0337] M and z

[0338] It is preferred that M is a monovalent pharmaceutically acceptable cation, and is more preferably Na+.

[0339] z is preferably 3.

[0340] Preferred conjugates of the first aspect of the present invention may have a DL of formula Ia:



Ia

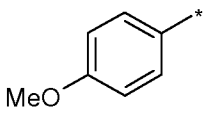
[0341] where

RL1', R20 and R21 are as defined above;

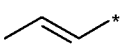
n is 1 or 3;

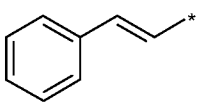
R1a is methyl or phenyl; and

R2a is selected from:

(a)  ;

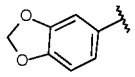
(b)  ;

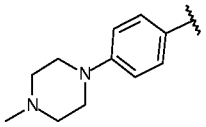
(c)  ;

(d)  ;

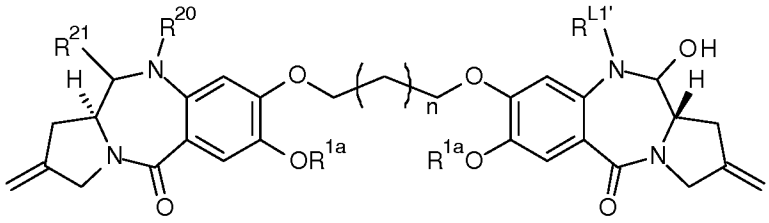
(e)  ;

(f)  ;

(g)  ; and

(h)  .

[0342] Preferred conjugates of the first aspect of the present invention may have a DL of formula Ib:



Ib

where

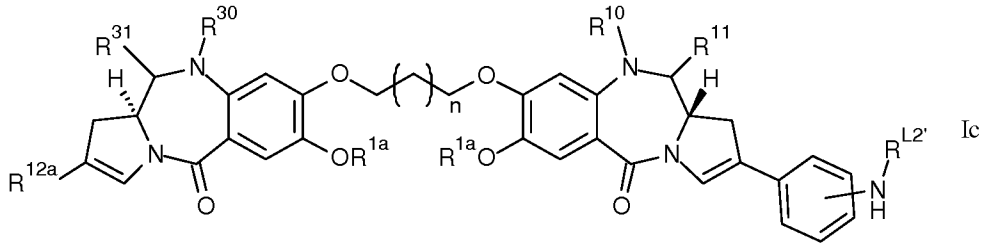
[0343] RL1', R20 and R21 are as defined above;

[0344] n is 1 or 3; and

[0345] R1a is methyl or phenyl.

[0346]

[0347] Preferred conjugates of the first aspect of the present invention may have a DL of formula Ic:

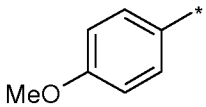


Ic

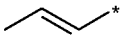
where RL2', R10, R11, R30 and R31 are as defined above

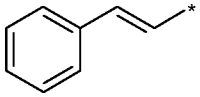
n is 1 or 3;

R12a is selected from:

(a)  ;

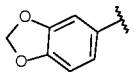
(b)  ;

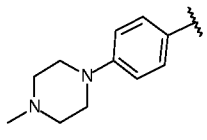
(c)  ;

(d)  ;

(e)  ;

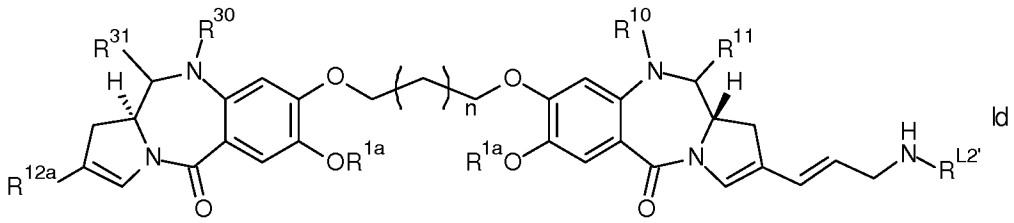
(f)  ;

(g)  ; and

(h)  .

the amino group is at either the meta or para positions of the phenyl group.

[0348] Preferred conjugates of the first aspect of the present invention may have a DL of formula Id:

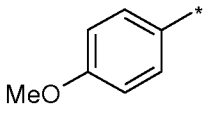

Id

where RL2', R10, R11, R30 and R31 are as defined above

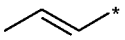
n is 1 or 3;

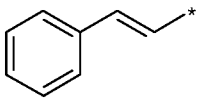
R1a is methyl or phenyl;

R12a is selected from:

(a)  ;

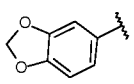
(b)  ;

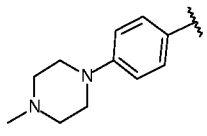
(c)  ;

(d)  ;

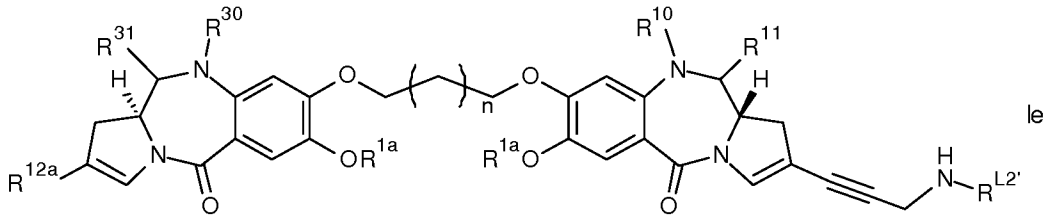
(e)  ;

(f)  ;

(g)  ; and

(h)  .

[0349] Preferred conjugates of the first aspect of the present invention may have a DL of formula Ie:



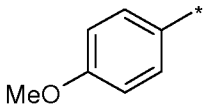
Ie

where RL2', R10, R11, R30 and R31 are as defined above

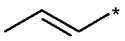
n is 1 or 3;

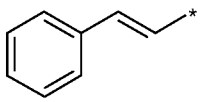
R1a is methyl or phenyl;

R12a is selected from:

(a)  ;

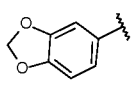
(b)  ;

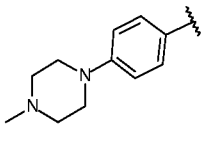
(c)  ;

(d)  ;

(e)  ;

(f)  ;

(g)  ; and

(h)  .

Antibody-Imaging Agent Conjugates

[0350] Antibodies and antigen binding fragment thereof s described herein may be conjugated to an imaging agent to form an antibody-imaging agent conjugate. The use of the phrase antibody-imaging agent conjugate specifically contemplates the use of a antigen binding fragment thereof of an antibody as the antibody portion in the conjugate.

[0351] In one embodiment, the antibody-drug conjugate comprises an antibody as described herein and an imaging agent.

[0352] The agent may be an imaging agent, by which term is meant an agent which may be detected, whether in vitro or in vivo in the context of a tissue, organ or organism in which the agent is located. Examples of agents include those useful for imaging of tissues *in vivo* or *ex vivo*. In an embodiment, the imaging agent emits a detectable signal, such as visible light, electromagnetic radiation, or radioactivity. In one embodiment, the imaging agent is opaque to radiation, such as X-ray radiation. In another embodiment, the imaging agent may be detected by a color change. Secondary binding ligands may also be used as imaging agents. Other available imaging agents may be used.

[0353] In one embodiment, an imaging agent may be a radiolabel, an enzyme, a fluorescent label, a luminescent label, a bioluminescent label, a magnetic label, a biotin, a PET (positron emission tomography) label, a SPECT (single-photon emission computed tomography) label, an MRI (magnetic resonance imaging) label, or an ultrasound imaging agent.

[0354] Examples of fluorescent labels include, but are not limited to, fluorescein isothiocyanate (FITC) (e.g., 5-FITC), fluorescein amidite (FAM) (e.g., 5-FAM), eosin, carboxyfluorescein, erythrosine, Alexa Fluor® (e.g., Alexa 350, 405, 430, 488, 500, 514, 532, 546, 555, 568, 594, 610, 633, 647, 660, 680, 700, or 750), carboxytetramethylrhodamine (TAMRA) (e.g., 5,-TAMRA), tetramethylrhodamine (TMR), and sulforhodamine (SR) (e.g., SR101).

[0355] In some embodiments, radiolabels include ^{3}H, ^{11}C, ^{13}N, ^{14}C, ^{15}N, ^{15}O, ^{35}S, ^{18}F, ^{32}P, ^{33}P, ^{47}Sc, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{75}Se, ^{76}Br, ^{77}Br, ^{86}Y, ^{89}Zr, ^{90}Y, ^{94}Tc, ^{95}Ru, ^{97}Ru, ^{99}Tc, ^{103}Ru, ^{105}Rh, ^{105}Ru, ^{107}Hg, ^{109}Pd, ^{111}Ag, ^{111}In, ^{113}In, ^{121}Te, ^{122}Te, ^{123}I, ^{124}I, ^{125}I, ^{125}Te, ^{126}I, ^{131}I, ^{131}In, ^{133}I, ^{142}Pr, ^{143}Pr, ^{153}Pb, ^{153}Sm, ^{161}Tb, ^{165}Tm, ^{166}Dy, ^{166}Ho, ^{167}Tm, ^{168}Tm, ^{169}Yb, ^{171}Lu, ^{177}Lu,^{186}Re, ^{188}Re, ^{189}Re, ^{197}Pt, ^{198}Au, ^{199}Au, ^{201}Tl, ^{203}Hg, ^{211}At, ^{212}Bi, ^{212}Pb, ^{213}Bi, ^{223}Ra, ^{224}Ac, and ^{225}Ac.

[0356] In an embodiment, the imaging agent is a contrast dye. For example, an MRI contrast agent can comprise a paramagnetic contrast agent (such as a gadolinium compound), a superparamagnetic contrast agent (such as iron oxide nanoparticles), a diamagnetic agent (such as barium sulfate), and combinations thereof. Metal ions preferred for MRI include those with atomic numbers 21-29, 39-47, or 57-83, and, more typically, a paramagnetic form of a metal ion with atomic numbers 21-29, 42, 44, or 57-83. Particularly preferred paramagnetic metal ions are selected from the group consisting of Gd(III), Fe(III), Mn(II and III), Cr(III), Cu(II), Dy(III), Tb(III and IV), Ho(III), Er(III), Pr(III) and Eu(II and III). Gd(III) is particularly useful. Note that as used herein, the term “Gd” is meant to convey the ionic form of the metal gadolinium; such an ionic form can be written as GD(III), GD3+, etc. with no difference in ionic form contemplated. A CT contrast agent can comprise iodine (ionic or non-ionic formulations), barium, barium

sulfate, Gastrografin (a diatrizoate meglumine and diatrizoate sodium solution), and combinations thereof. In another embodiment, a PET or SPECT contrast agent can comprise a metal chelate.

[0357] Non-limiting examples of paramagnetic ions of potential use as imaging agents include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being particularly preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to lanthanum (1H), gold (III), lead (H), and especially bismuth (III).

[0358] In certain embodiments, the claimed proteins or peptides may be linked to a secondary binding ligand or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of suitable enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase and glucose oxidase. Preferred secondary binding ligands are biotin and avidin or streptavidin compounds.

[0359] Ultrasound imaging agents include gas microbubbles, such as gas-filled lipophilic or lipid-based bubbles or gas or liquid entrapped in porous inorganic particles that facilitate microbubble release upon delivery to a subject. Gases, liquids, and combinations thereof suitable for use with the presently disclosed subject matter include air; nitrogen; oxygen; is carbon dioxide; hydrogen; nitrous oxide; an inert gas such as helium, argon, xenon or krypton; a sulfur fluoride such as sulfur hexafluoride, disulfur decafluoride or trifluoromethylsulfur pentafluoride; selenium hexafluoride; an optionally halogenated silane such as tetramethylsilane; a low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms), for example an alkane such as methane, ethane, a propane, a butane or a pentane, a cycloalkane such as cyclobutane or

cyclopentane, an alkene such as propene or a butene, or an alkyne such as acetylene; an ether; a ketone; an ester; a halogenated low molecular weight hydrocarbon (e.g. containing up to 7 carbon atoms); or a mixture of any of the foregoing. Halogenated hydrocarbon gases can show extended longevity, and thus are preferred for some applications. Representative gases of this group include decafluorobutane, octafluorocyclobutane, decafluoroisobutane, octafluoropropane, octafluorocyclopropane, dodecafluoropentane, decafluorocyclopentane, decafluoroisopentane, perfluoropexane, perfluorocyclohexane, perfluoroisohexane, sulfur hexafluoride, and perfluorooctaines, perfluorononanes; perfluorodecanes, optionally brominated.

[0360] In still further embodiments, a targeting moiety may be operatively coupled to a nanoparticle. Nanoparticles include, but are not limited to colloidal gold and silver nanoparticles. Metal nanoparticles exhibit colors in the visible spectral region. It is believed that these colors are the result of excitation of surface plasmon resonances in the metal particles and are extremely sensitive to size, shape, and aggregation state of particles; dielectric properties of the surrounding medium; adsorption of ions on the surface of the particles.

Conjugation

[0361] Antibodies and antigen fragments therof disclosed herein may be conjugated to drugs or imaging agents using site-specific or non-site specific methods of conjugation.

[0362] In an embodiment antibodies, and antigen fragments therof, can be non-site specifically conjugated to drugs or imaging agents by partial reduction of the antibody followed by reaction with a desired drug or imaging agent with or without a linker moiety attached. The antibody may be reduced using DTT or similar reducing agent. The drug or imaging agent with or without a linker moiety attached can then be added at a molar excess to the reduced antibody in the presence of DMSO. After conjugation, excess free cysteine may be added to quench

unreacted drug or imaging agent. The reaction mixture may then be purified and buffer-exchanged into PBS.

[0363] In another embodiment, antibodies, and antigen fragments therof, can be conjugated using site specific conjugation. The site specific conjugation may be through a cysteine, residue or a non-natural amino acid.

[0364] In one embodiment, the cytotoxic or imaging agent is conjugated to the antibody or antigen binding fragment thereof through at least one cysteine residue. In one embodiment, the cytotoxic or imaging agent is conjugated to the antibody or antigen binding fragment thereof through a cysteine substitution of at least one of positions 239, 248, 254, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 440, 441, 442, 443 and 446, wherein the numbering corresponds to the EU index in Kabat.

[0365] In one embodiment a cysteine is inserted in the antibody or antigen binding fragment thereof between positions 239 and 240, wherein the numbering corresponds to the EU index in Kabat. Such an insetion may be made in combination with one or more substitutions

[0366] In one embodiment, the cytotoxic or imaging agent is conjugated to the antibody or antigen binding fragment thereof through a thiol-maleimide linkage.

[0367] Since the antibody or antigen binding fragment thereof serves as a targeting agent, one molecule of the drug or imaging agent may be conjugated to each antibody or antigen binding fragment thereof or multiple molecules of the drug or imaging agent may be conjugated if it does not present steric hindrance to the antibody or antigen binding fragment thereof thereof. In one embodiment, two molecules of the drug or imaging agent may be conjugated to each antibody. For example, in one embodiment, one molecule may be conjugated to each heavy

chain. In another embodiment, four molecules of the drug or imaging agent may be conjugated to each antibody. For example, in one embodiment, two molecules may be conjugated to each heavy chain.

[0368] In one embodiment, the antibody or antigen binding fragment thereof may be directly conjugated to the drug or imaging agent. In another embodiment, the antibody or antigen binding fragment thereof may be conjugated through a linker. The linker may be as disclosed above in relation to pyrrolobenzodiazepines. It will be appreciated by the person skilled in the art that such linkers may also be used with a variety of other cytotoxic and imaging agents.

Methods of Use

[0369] Antibody-drug conjugates may be administered orally or via injections (including subcutaneous injection, intradermal injection, intramuscular injection, intravenous injection, a chemotherapy port, or a peripherally inserted central catheter). The dose of the antibody-drug conjugate depends on the drug chosen for the antibody-drug conjugate. Because the antibody or antigen binding fragment thereofacts as a targeting agent, the same dose of drug as administered without being conjugated to the antibody or antigen binding fragment thereofmay be used or a reduced dose of drug compared to that administered without the antibody or antigen binding fragment thereof . Conjugation to the antibody may reduce side effects, increase efficacy, and/or allow for a reduced dosage. It may also allow for administration of a drug that might otherwise be too toxic.

[0370] If a reduced dosage is a benefit of the antibody-drug conjugate, the dosage may be reduced by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, or 80%.

[0371] For therapeutic application, antibody-drug conjugates are typically combined with a pharmaceutically acceptable carrier, adjuvant, diluent and/or excipient. In one embodiment, the

same formulation is used as the drug when administered alone. In one embodiment, the antibodies or antigen binding fragment thereof s are combined with water for injection. In another embodiment, they are prepared in a sterile, preservative-free liquid solution with histidine, glycine, and chloride. In another embodiment, examples of suitable carriers for instance comprise keyhole limpet haemocyanin (KLH), serum albumin (e.g. BSA or RSA) and ovalbumin. Many suitable adjuvants, oil-based and water-based, are known to a person skilled in the art. In one embodiment said adjuvant comprises Specol. In another embodiment, said suitable carrier comprises a solution like for example saline. In other embodiments, the antibodies or antigen binding fragment thereof s are provided in a lyophilized form and mixed with water for injection prior to administration.

Methods of Detection Using Antibody-Imaging Agent Conjugates

[0372] The antibody-imaging agent conjugates described herein may be used in a variety of *in vivo*, *in vitro* and *ex vivo* methods of detection of cancer cells expressing the 5T4 cell surface antigen. Methods include administering to a subject or applying to a sample a composition comprising the antibody-imaging agent conjugate and detecting the imaging agent to thereby detect cancer cells expressing 5T4.

[0373] If cells are positive for 5T4 expression, this means that an antibody-drug conjugate targeting 5T4 will offer improved results in methods of killing cancer cells or methods of treating cancer. These methods may also be used to detect the location of cancer cells in a patient, as part of diagnosis, staging of cancer, or as a tool in developing a treatment plan. The antibody-imaging agent conjugates further may be used to detect borders between cancer cells and noncancer cells, whether *in vitro*, *ex vivo*, or *in vivo* (such as during a surgical resection of a solid tumor).

[0374] The antibody-imaging agents may be used in a variety of techniques, including, but not limited to, western blotting, ELISA assays, immunofluorescence assays, immunohistochemistry assays, and flow cytometry. The antibody-imaging agents may also be used in X-ray, ultrasound, PET, SPECT, or MRI scans.

[0375] An antibody-imaging agent may be applied locally or it may be administered systemically such as orally or by injection (including subcutaneous injection, intradermal injection, intramuscular injection, intravenous injection, a chemotherapy port, or a peripherally inserted central catheter).

[0376] Reference will now be made in detail to the present exemplary embodiments, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers will be used throughout the drawings to refer to the same or like parts. Other embodiments will be apparent to those skilled in the art from consideration of the specification and practice disclosed herein. The embodiments are further explained in the following examples. These examples do not limit the scope of the claims, but merely serve to clarify certain embodiments. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit being indicated by the following claims.

EXAMPLES

Example 1 - Isolation and characterization of the anti-5T4 antibody ZY07N8-A07

Immunizations and generation of hybridomas

[0377] While several 5T4-specific IgGs were obtained from different immunization strategies the parental clone ZY07N8-A07 was isolated from mouse 4 following these methods. 8 VelocImmune II mice (Regeneron, Tarrytown, NY) were immunized with human 5T4-HIS10

FLAG protein (generated in-house) using a rest boost immunization method according to the schedule outlined below:

Day 0: 10µg human 5T4-His10 FLAG protein in Freunds complete adjuvant (FCA) 100µl per site x two sites subcutaneous

Day 14 10µg human 5T4-His10 FLAG protein in Freunds incomplete adjuvant (IFA) 100µl per site x two sites subcutaneous

Day 28 10µg human 5T4-His10 FLAG protein in IFA 100µl per site x two sites subcutaneous

Day 42 10µg human 5T4-His10 FLAG protein in IFA 100µl per site x two sites subcutaneous

Day 45 10µg human 5T4-His10 FLAG protein in PBS 200µl intraperitoneally

Day 49 Endpoint tissue harvest

[0378] Sera were collected from mice on days -4, 34 and 38 and anti-5T4 antibody titres determined in an ELISA assay. Flat bottom 96 well plates (Nunc Maxisorp) were coated with 5µg/ml human 5T4-His10 FLAG. Antibodies against 5T4 were detected with goat anti-mouse IgG γ chain specific antibody conjugated to horseradish peroxidase (Sigma#A3673). Immunized mice were sacrificed by anesthetic overdose and the draining lymph nodes and spleens removed. Cells were dissociated from the tissue by mechanical disruption, suspended in DMEM and counted. 2.3 x 10^7 lymph node cells and 5 x 10^7 spleen cells were mixed together giving a total of 7.3 x 10^7 cells from mouse 4. Cells were mixed with 36.5 x 10^7 myeloma SP2 cells purchased from ATCC (#CRL-1581). Fusions were performed using polyethylene glycol (1). The resulting fusion mixture was plated into 20 plates of 88 wells per plate and cultured in selective DMEM media (DMEM high glucose (Gibco#41966), 20% FCS (Sigma#**12133C**), 4mM Glutamine

(Gibco#25030), 100U/100ug/ml Penicillin/Streptomycin (Invitrogen#15140-122), 10% Hybridoma cloning factor (Roche#10879100), 2% oxaloacetate/pyruvate/insulin (Sigma#O5003), 2% Hypoxanthine/Azaserine (Sigma#A9666)). Hybridomas were grown in the selective media before supernatants were harvested from wells 13 days post fusion. Supernatants were tested in various assays as described in the primary screening section of this document. Hybridomas meeting the selection criteria were picked from the fusion plates and cryopreserved. Hybridoma mRNA for heavy and light chain variable regions were isolated from hits using Oligo d(T) magnetic beads (Novagen#69593) in combination with the kingfisher 96 automated magnetic separator (Thermo scientific). Purified RNA was transcribed to cDNA using superscript III reverse transcriptase (Invitrogen#18080085) at 44˚C for 1 hr and then tailed with poly(G) by incubation with excess dGTP and terminal transferase (NEB#M0315L) at 37˚C for 1 hr. Variable light and heavy chain genes were amplified using Taq polymerase (Thermo#SP0956/b) in separate reactions using poly(C) and specific primers to either the constant heavy 1 or kappa constant domains. Chain termination method was used to sequence the PCR product. Well lines were expanded into 24 well plates and allowed to overgrow in serum free media (HL-1 (Lonza#77201), HyberZero (Statens Institute#71800)) for 10 days. Supernatant were harvested and purified using Protein A Phynexus tips (Phynexus#PTZ92-20-01). Purified IgGs were rescreened for activity. 22 hybridoma well lines including ZY07N8-A07 were selected for cellular cloning using limiting dilution based on primary screen and sequence data. Following 10 days of growth 16 clones from each well line had the culture supernatant harvested and tested for activity. 4 clones from each well line were selected for propagation. Sequence was reconfirmed from these clonal populations before selecting one for 50 ml overgrow in serum free media. Supernatants were collected and purified using protein G columns. Purified monoclonal

IgGs were tested in various binding and biological assays as described in following sections of this document.

[0379] *See* (1) Kohler and Milstein, Continuous culture of fused cells secreting antibody of a predefined specificity. Nature 1975;256:495-497.

Primary screening

[0380] Hybridoma supernatants were tested to assess the ability of any chimeric antibodies they might contain to directly bind isolated recombinant human and cyno 5T4 ECD Fc (generated in-house), via Homogeneous Time Resolved Flurorescence (HTRF). 5% hybridoma supernatant was combined with 1nM d2 Jackson goat anti-mouse Fc (Cisbio custom labelling), and a 1 in 2015 dilution of Eu-TBP-K labelled (Cisbio 62EUSPEA) human 5T4 ECD Fc, or a 1 in 1542 dilution of Eu-TBP-K labelled cyno 5T4 ECD Fc, and assay buffer (Gibco-Invitrogen Dulbecco's phosphate buffered saline 14190, containing Sigma 0.1% BSA A9576 and BDH 0.4M potassium fluoride 103444T) in Corning-Costar 384-well black, shallow, solid-bottom assay plates, type 3676. Non-specific binding (also referred to interchangeably as 'cryptate blank') was determined by combining 1nM d2 Jackson goat anti-mouse Fc, a 1 in 2015 dilution of Eu-TBP-K human 5T4 ECD Fc, or a 1 in 1542 dilution of Eu-TBP-K cyno 5T4 ECD Fc, hybridoma growth media and assay buffer, but no actual hybridoma supernatant. The total well volume was 20µl, and each reagent was added in 5µl (N.B. All quoted concentrations or dilutions are final concentrations or dilutions). The plates were sealed with 4titude adhesive plastic film (4ti-0510), lidded, covered in foil and incubated at room temperature for 4hrs.

[0381] Plates were then counted on a Perkin Elmer Envision plate reader, using excitation wavelength 320nm and emission wavelengths 620 and 665nm. The Envision measured any Fluorescence Resonance Energy Transfer (FRET) taking place between the Eu-TBP-K

'donor' (associated with 5T4), and the d2 'acceptor' (associated with antibody), following excitation of the donor, if the two were in close proximity as a result of successful binding of antibody and antigen. If antibody and antigen did not bind, then donor and acceptor were not in close enough proximity for FRET to be measured. Hits were defined as any hybridoma supernatant sample which gave a Delta F value of 100 or greater. Delta F was calculated as follows:-

i) Ratio = (counts at 665nm / counts at 620nm) x 10,000

ii) Delta F = ((Sample ratio – non-specific mean ratio) / non-specific mean ratio).

Table 2. Binding of hybridoma supernatant to Eu-TBP-K 5T4 ECD Fc using HTRF

Clone ID	Human 5T4 ECD Fc (Delta F)	Cyno 5T4 ECD Fc (Delta F)
ZY07N8-A07	400 (n=1)	456 (n=1)

[0382] Samples that bound Eu-TBP-K human 5T4 ECD Fc with values of 100 Delta F or greater were produced as Phynexus purified IgGs with a murine Fc, and re-evaluated for Eu-TBP-K human and cyno 5T4 ECD Fc binding at 0.003 – 3nM, using the direct binding HTRF model described above. They were also evaluated in a murine 5T4 ECD Fc direct binding assay, at a concentration range of 0.001 – 3nM, with the substitution of EU-TBP-K labelled murine 5T4 ECD Fc at a 1 in 684 dilution for the Eu-TBP-K-labelled human or cyno 5T4 antigen. Data was analysed as above and any concentration-dependant binding responses elicited plotted using Graphpad Prism software, Log Conc (M) vs Delta F. Curves were fitted using non-linear regression, and EC50 values determined. Figure 3 shows Binding of ZY07N8-A07 as a Phynexus purified chimeric antibody (murine Fc) to Eu-TBP-K human and cyno 5T4 ECD Fc using HTRF.

Table 3. EC50 data for binding of ZY07N8-A07 as a Phynexus purified chimeric

antibody (murine Fc) to Eu-TBP-K human and cyno 5T4 ECD Fc using HTRF		
Clone ID	Human 5T4 ECD Fc EC50 (nM)	Cyno 5T4 ECD Fc EC50 (nM)
ZY07N8-A07	0.089 (n=1)	0.21 (n=1)

[0383] Hits were also grown up as single cell clones/well lines, then re-evaluated for Eu-TBP-K human 5T4 ECD Fc binding using the previously described HTRF-based direct binding assay. Each well line was evaluated at three concentrations, 5, 10 and 25% final. Samples with varying degrees of activity (including some <100 Delta F) were taken forward and produced as purified chimeric monoclonal antibodies with a murine Fc. N.b. The reason for progression of samples with low Delta F values was to retain as much diversity as possible in the lead panel, with the expectation activity might later improve when samples were eventually purified and reformatted to full human IgGs.

Table 4. Active well lines (shown in bold) of clone ZY07N8-A07 in the Eu-TBP-K human 5T4 ECD Fc HTRF-based binding assay			
Example Data (n=1)			
Clone	**ZY07N8-A07**	**ZY07N8-A07**	**ZY07N8-A07**
Sample Conc.	**25%**	**10%**	**5%**
Well line 1	**329**	**576**	**747**
2	55	**158**	**311**
3	40	**130**	**245**
4	**266**	**560**	**747**
5	**231**	**556**	**721**
6	49	**141**	**363**

7	67	**213**	**495**
8	**229**	**467**	**771**
9	**317**	**631**	**734**
10	71	**258**	**459**
11	**154**	**389**	**625**
12	25	**111**	**180**
13	96	**255**	**445**
14	45	**185**	**302**
15	65	**181**	**339**
16	56	**167**	**329**
no of active well lines	**6/16**	**16/16**	**16/16**

[0384] Purified chimeric monoclonal antibodies with a murine Fc were titrated at 0.001 to 3nM for binding to Eu-TBP-K human, cyno and murine 5T4 ECD Fc using the previously described direct binding assays. Data was analysed as previously described and any concentration-dependent binding responses elicited plotted using Graphpad Prism software, Log Conc (M) vs Delta F. Curves were fitted using non-linear regression, and EC50 values determined. Figure 4 shows an example of binding of ZY07N8-A07 purified chimeric mab with murine Fc to Eu-TBP-K 5T4 ECD FC using HTRF.

Table 5. EC50 data from example binding of ZY07N8-A07 purified chimeric mab with murine Fc to Eu-TBP-K 5T4 ECD FC using HTRF

Clone ID	Human 5T4 ECD Fc Binding	Cyno 5T4 ECD Fc Binding	Murine 5T4 ECD Fc Binding
	EC50 (nM)	EC50 (nM)	EC50 (nM)
ZY07N8-A07	0.032 (n=1)	0.037 (n=1)	no binding (n=1)

Table 6. Mean EC50 data for binding of ZY07N8-A07 purified chimeric mab with murine Fc to Eu-TBP-K 5T4 ECD FC using HTRF

Clone ID	Human 5T4 ECD Fc Binding Mean EC50 (nM)	Cyno 5T4 ECD Fc Binding Mean EC50 (nM)	Murine 5T4 ECD Fc Binding Mean EC50 (nM)
ZY07N8-A07	0.050 +/- 0.0192 (n=3)	0.059 +/- 0.0256 (n=3)	no binding (n=3)

Re-formatting to human IgG1 and confirmation of binding to 5T4

[0385] Samples were reformatted to purified human IgG1 monoclonal antibodies and titrated at 0.001 to 3nM for binding to Eu-TBP-K human 5T4 ECD Fc using the direct binding assay described earlier, but with Alexa647 (Molecular Probes A-20186) labelled goat anti human kappa light chain detection at 3nM substituted for d2 Jackson goat anti-mouse detection at 1nM. Data was analysed as previously reported and any concentration-dependant binding responses elicited plotted using Graphpad Prism software, Log Conc (M) vs Delta F. Curves were fitted using non-linear regression and EC50 values determined. Figure 5 shows binding of ZY07N8-A07 as a purified human mab to Eu-TBP-K human 5T4 ECD Fc using HTRF.

Table 7. EC50 data from example binding of ZY07N8-A07 purified human mab to Eu-TBP-K human 5T4 ECD FC using HTRF	
Clone ID	Human 5T4 ECD Fc Binding EC50 (nM)
ZY07N8-A07	0.071 (n=1)

Table 8. Mean EC50 data for binding of ZY07N8-A07 purified human mab to Eu-TBP-K human 5T4 ECD FC using HTRF	
Clone ID	Human 5T4 ECD Fc Binding Mean EC50 (nM)
ZY07N8-A07	0.091 (n=2)

Specificity/selectivity of ZY07N8-A07 by DELFIA binding assay

[0386] A BLAST search of human related-antigens to the extracellular domain of human 5T4 flagged up only proteins with low amino acid identity (i.e. <33%). Of these 3 were commercially available and will be used to check the specificity of A07 by ELISA: Platelet glycoProtein Ib alpha, 31% identity; Platelet glycoProtein V, 30% identity; Leucine-rich repeat-containing protein 4C (netrin-G1 ligand 1 or NGL-1), 27% identity (all from R&D systems). Human 5T4 Flag-His was used to show positive binding and CD86 Flag-His was used as a negative control with the same tags (both produced in-house). Mouse 5T4 Flag-His was also used to check for mouse cross-reactivity. Antigens were coated on NUNC MaxiSorp plates at 5ug/mL to saturate the wells. The 5T4-A07 human IgG1 antibody was then incubated at various concentrations ranging from 10 to 0.1µg/mL. Bound antibodies were detected using a mouse

anti-human secondary antibody labelled with Europium (Perkin Elmer). Each point has been done in triplicate. ZY07N8-A07 IgG shows binding to human 5T4, but not mouse 5T4 or any of the others proteins used. Figure 6 shows specificity ELISA ZY07N8-A07 demonstrating specific binding to human 5T4.

Example 2 - Lead optimization of anti-5T4 antibody 5T4-A07

Germlining of ZY07N8-A07 and conversion to scFv format

[0387] The amino acid sequence of the V_H and V_L domains for ZY07N8-A07 were aligned to the known human germline sequences in the VBASE database (1), and the closest germline was identified by sequence similarity. For the V_H domain this was Vh3-15 (DP-38) and JH6, for the V_L domain it was Vκ1 A30 and JK1. Except for Vernier residues (2), which were left unchanged, the germlining process involved reverting framework residues in the V_H and V_L domains to these closest human germline. For ZY07N8-A07, no residues required changing in the V_L domain, but a total of 2 were required in the V_H domain. These were at Kabat positions 82a, where isoleucine (I) was reverted to asparagine (N), and 105, where histidine (H) was reverted to glutamine (Q). These changes were made using standard site directed mutagenesis techniques with the appropriate mutagenic primers. Both the germlined and original non-germlined ZY07N8-A07, were re-formatted to single chain variable fragment (scFv) format in preparation for affinity optimization using phage display. The variable heavy (V_H) and variable light (V_L) domains were amplified separately from their respective IgG expression vectors with the addition of specific cloning sites and a flexible linker region. Recombinatorial PCR was then performed to generate a complete scFv construct, which was cloned into the pCantab6 phagemid vector (3). There was no difference in ability of both purified scFv to inhibit binding of 5T4-A07 parent IgG binding to human 5T4, as demonstrated in the epitope competition assay.

[0388] *See* (1) Tomlinson, I., VBASE. 1997, MRC Centre of Protein Engineering, Cambridge, UK; Journal of Molecular Biology 224:487-499; (2) Foote, J., et al. J Mol Biol, 1992. 224: p. 487; (3) Hutchings, C., Generation of Naïve Human Antibody Libraries, in Antibody Engineering, R. Kontermann and S. Dubel, Editors. 2001, Springer Laboratory Manuals, Berlin. p. 93.

Optimization of ZY07N8-A07 scFv by targeted mutagenesis

[0389] Germlined ZY07N8-A07 (ZY07N8-A07 GL) was optimized using affinity-based phage selections. Large scFv libraries derived from the lead scFv sequence were created by oligonucleotide-directed mutagenesis of the variable heavy (VH) complementarity determining regions 1 or 2 (CDR1 or CDR2) or variable light (VL) chain complementarity determining regions 1, 2 or 3 (CDR1, 2 or 3) using standard molecular biology techniques as described (1). The libraries were subjected to affinity-based phage display selections in order to select variants with a higher affinity to human 5T4. The selections were performed essentially as described previously (2). In brief, the scFv-phage particles were incubated in solution with recombinant biotinylated human 5T4 Fc (generated using in-house produced material and biotinylated via free amines using EZ LINK™ Sulfo-NHS-LC-Biotin (Thermo/Pierce, product: 21335)). ScFv bound to antigen were then captured on streptavidin-coated paramagnetic beads (DYNABEADS® M-280) following manufacturer's recommendations. The selected scFv-phage particles were then rescued as described previously (3), and the selection process was repeated in the presence of decreasing concentrations of biotinylated human 5T4 (a typical example would be 100 nM to 10 pM over four rounds). Crude scFv-containing periplasmic extracts were prepared for a representative number of individual scFv from the CDR-targeted selection outputs and screened in an epitope competition HTRF® (Homogeneous Time-Resolved Fluorescence) assay format

against the parent antibody ZY07N8-A07 (as described below in Example 2C). Hits identified in the screen, i.e. scFv variants which showed a significantly improved inhibitory effect when compared to parent ZY07N8-A07, were subjected to DNA sequencing, and unique variants from variable heavy CDR1 or CDR2 and variable light library CDR1, CDR2 or CDR3 outputs were produced as purified scFv and tested. To generate further improvement, CDR-randomized selection outputs comprising of large numbers of scFv variants with the ability to inhibit the binding of parent ZY07N8-A07 to human 5T4 were recombined to form libraries in which clones contained randomly paired individually randomized VH CDR1, VH CDR2, VL CDR1, VL CDR2 or VL CDR3. Further phage display selections were performed using decreasing concentrations of biotinylated 5T4 (typically from 1nM to 1pM in four rounds).

[0390] *See* (1) Clackson, T. and Lowman, H.B. Phage Display – A Practical Approach, 2004. Oxford University Press; (2) Thompson, J. et al. J Mol Biol. 256(1):77-88, 1996; (3) Osbourn, J.K. et al. Immunotechnology, 2(3):181-96, 1996.

Table 9A-F: Amino acid usage for all putative hits identified in epitope competition assay as crude periplasmic scFv

	5T4-A07	5T4_0108	Amino acids used (% frequency)							
VH-CDR1 (n=19) - IMGT defined	G	G	G (100%)							
	F	F	F (84.2%)	D (10.5%	E (5.3%)					
	T	T	T (84.2%)	W (10.5%)	G (5.3%)					
	F	F	F (84.2%)	A (5.3%)	S (5.3%)	T (5.3%)				
	S	S	S (26.3%)	A (26.3%)	Q (15.8%)	H (10.5%)	T (10.5%)	G (5.3%)	K (5.3%)	
	N	N	N (36.8%)	T (21.1%)	Q (10.5%)	R (10.5%)	A (5.3%)	L (5.3%)	P (5.3%)	S (5.3%)
	A	A	Q (26.3%)	Y (26.3%)	A (21.1%)	L (10.5%)	F (5.3%)	M (5.3%)	S (5.3%)	
	W	W	P (52.6%)	W (21.1%)	Q (15.8%)	L (10.5%)				
	M	M	M (57.9%)	L (21.1%)	I (10.5%)	G (5.3%)	V (5.3%)			
	S	S	H (47.4%)	S (42.1%)	T (10.5%)					

	5T4-A07	5T4_0108	Amino acids used (% frequency)									
VH-CDR2 (n=29)	R	R	R (82.8%)	A (10.3%)	Q (6.9%)							
	I	I	I (58.6%)	V (31%)	L (3.4%)	M (3.4%)	Q (3.4%)					
	R	R	R (79.3%)	K (20.7%)								
	S	A	A	S (41.4%)	G (3.4%)							

			(55.2%)									
	K	**Q**	Q (24.1%)	K (20.7%)	E (13.8%)	S (10.3%)	V (10.3%)	I (6.9%)	H (3.4%)	L (3.4%)	R (3.4%)	Y (3.4%)
	A	**R**	A (37.9%)	Q (34.5%)	R (13.8%)	L (6.9%)	M (6.9%)					
	D	D	D (100%)									
	G	G	G (86.2%)	N (6.9%)	E (3.4%)	L (3.4%)						
	G	G	G (86.2%)	A (3.4%)	D (3.4%)	E (3.4%)	Q (3.4%)					
	T	T	T (82.8%)	E (3.4%)	Q (3.4%)	R (3.4%)	V (3.4%)	W (3.4%)				
	T	T	T (79.3%)	Q (6.9%)	V (6.9%)	I (3.4%)	P (3.4%)					
	D	D	D (79.3%)	Q (6.9%)	Y (6.9%)	F (3.4%)	W (3.4%)					
	S	S	S (100%)									
	A	A	A (100%)									
	A	A	A (100%)									
	P	P	P (100%)									
	V	V	V (100%)									
	K	K	K (100%)									
	G	G	G (100%)									

	5T4-A07	**5T4_0108**	**Amino acids used (% frequency)**											
VL-CDR2 (n=35)	A	A	A (71.4%)	N (28.6%)										
	A	**G**	A (51.4%)	G (25.7%)	S (17.1%)	T (2.9%)	V (2.9%)							
	S	**W**	S (31.4%)	W (28.6%)	D (17.1%)	G (5.7%)	N (5.7%)	Q (5.7%)	H (2.9%)	T (2.9%)				
	S	**Q**	R (28.6%)	Q (25.7%)	T (20%)	S (11.4%)	H (5.7%)	K (2.9%)						
	L	**R**	R (28.6%)	L (25.7%)	P (11.4%)	W (5.7%)	H (2.9%)	K (2.9%)	M (2.9%)	N (2.9%)	Q (2.9%)	S (2.9%)	T (2.9%)	V (2.9%)
	Q	**D**	D (50%)	M	S	V								

				(16.7%)	(16.7%)	(16.7%)								
	S	S	S (100%)											

	5T4-A07	5T4_0108	Amino acids used (frequency)											
VL-CDR3 (n=147)	L	L	L (99.3%)	V (0.7%)										
	Q	Q	Q (100%)											
	Q	Q	Q (84.4%)	M (15%)	L (0.7%)									
	N	**Y**	R (57.8%)	K (23.8%)	Y (10.9%)	F (2%)	N (2%)	A (1.4%)	H (0.7%)	T (0.7%)	W (0.7%)			
	S	**R**	R (60.5%)	K (31.3%)	A (2%)	N (2%)	Q (1.4%)	L (0.7%)	M (0.7%)	S (0.7%)	T (0.7%)			
	Y	**V**	M (35.4%)	T (35.4%)	V (14.3%)	A (2.7%)	I (2.7%)	Q (2.7%)	Y (2%)	L (1.4%	P (1.4%)	K (0.7%)	R (0.7%)	S (0.7%)
	P	P	P (100%)											
	W	W	W (100%)											
	T	T	T (87.1%)	R (6.8%)	M (2%)	V (2%)	A (0.7%)	K (0.7%)	S (0.7%)					

Table 10A-F: Amino acid usage for all confirmed hits (>5-fold improved over parent) identified in epitope competition assay as purified scFv

	5T4-A07	5T4_0108	Amino acids used (% frequency)				
VH-CDR1 (n=8) - IMGT defined	G	G	G (100%)				
	F	F	F (62.5%)	D (25.0%)	E (12.5%)		
	T	T	T (62.5%)	W (25.0%)	G (12.5%)		
	F	F	F (62.5%)	A (12.5%)	S (12.5%)	T (12.5%)	
	S	S	S (50.0%)	H (12.5%)	K (12.5%)	Q (12.5%)	T (12.5%)
	N	N	N (62.5%)	L (12.5%)	S (12.5%)	T (12.5%)	
	A	A	A (37.5%)	Y (25.0%)	L (12.5%)	M (12.5%)	Q (12.5%)
	W	W	W (37.5%)	P (37.5%)	L (25.0%)		
	M	M	M (75.0%)	I (12.5%)	L (12.5%)		
	S	S	S (62.5%)	H (37.5%)			

	5T4-A07	5T4_0108	Amino acids used (% frequency)		
VH-CDR2 (n=5)	R	R	R (80%)	Q (20%)	
	I	I	I (100%)		
	R	R	R (80%)	K (20%)	

	S	**A**	A (100%)		
	K	**Q**	Q (40%)	S (40%)	V (20%)
	A	**R**	R (40%)	Q (40%)	A (20%)
	D	D	D (100%)		
	G	G	G (100%)		
	G	G	G (100%)		
	T	T	T (100%)		
	T	T	T (100%)		
	D	D	D (100%)		
	S	S	S (100%)		
	A	A	A (100%)		
	A	A	A (100%)		
	P	P	P (100%)		
	V	V	V (100%)		
	K	K	K (100%)		
	G	G	G (100%)		

	5T4-A07	**5T4_0108**	**Amino acids used (% frequency)**			
VL-CDR2 (n=6)	A	A	A (66.7%)	N (33.3%)		
	A	**G**	G (66.7%)	A (16.7%)	S (16.7%)	
	S	**W**	W (66.7%)	S (16.7%)	G (16.7%)	
	S	**Q**	Q (50%)	T (33.3%)	R (16.7%)	

	L	**R**	R (66.7%)	L (16.7%)	P (16.7%)	
	Q	**D**	D (50%)	M (16.7%)	S (16.7%)	V (16.7%)
	S	S	S (100%)			

	5T4-A07	**5T4_0108**	**Amino acids used (% frequency)**					
VL-CDR3 (n=27	L	L	L (100%)					
	Q	Q	Q (100%)					
	Q	Q	Q (81.5%)	M (14.8%)	L (3.7%)			
	N	**Y**	R (63%)	Y (11.1%)	K (11.1%)	F (7.4%)	H (3.7%)	N (3.7%)
	S	**R**	R (63%)	K (25.9%)	N (7.4%)	A (3.7%)		
	Y	**V**	M (40.7%)	T (29.6%)	V (22.2%)	I (3.7%)	P (3.7%)	
	P	P	P (100%)					
	W	W	W (100%)					
	T	T	T (92.6%)	M (3.7%)	V (3.7%)			

129

[0391] The VH-CDR3 was not targeted during lead optimisation, as antibodies derived from mouse immunisation hybridomas are often optimised in this region already through somatic hypermutation in vivo and generally targeting the VH-CDR3 does not result in antibodies with large improvements in binding affinity.

[0392] It is also of note that for the VL-CDR1, targeting this CDR during lead optimisation resulted in no significant improvements in potency versus the parent in the epitope competition assay. However, despite there being no significant improvements over parent, numerous amino acid changes within these regions resulted in antibodies that retained binding and specificity to 5T4, albeit with similar affinity to that of the parental antibody.

Identification of improved scFv using a parent antibody binding to 5T4 epitope competition assay

[0393] Periprep scFv were tested for their ability to compete biotinylated ZY07N8-A07 IgG for binding to Eu-TBP-K human 5T4 ECD Fc as follows:-

[0394] 25% final periprep scFv were preincubated with a 1 in 644 or 1 in 524 dilution of Eu-TBP-K human 5T4 ECD Fc for 15 minutes in Corning-Costar 384-well black assay plates. 0.5nM biotinylated ZY07N8-A07 IgG (labelled using Pierce EZ link Sulfo-NHS-LC-Biotin 21336) and 10nM Cisbo Streptavidin XL665 were then added. Total Binding wells contained biotinylated ZY07N8-A07 IgG, Eu-TBP-K human 5T4 ECD Fc, SAXL665, and periprep buffer, but no periprep active sample. Non-specific binding wells contained Eu-TBP-K human 5T4 ECD Fc, SAXL665, periprep buffer and assay buffer, but no periprep active sample and no biotinylated ZY07N8-A07 IgG. The total assay volume was 20ul/well, with each reagent being

added in 5µl. As detailed above, any shortfall was made up with assay buffer or periprep buffer (TES ph7.4), such that all wells contained the same final percentage of each buffer type.

[0395] Assay plates were lidded, covered in foil and incubated at room temperature for 4hrs, before being counted on a Perkin Elmer Envision plate reader using excitation wavelength 320nm and emission wavelengths 620 and 665nm. The Envision measured any Fluorescence Resonance Energy Transfer (FRET) taking place between the Eu-TBP-K 'donor' (associated with 5T4), and the SAXL665 'acceptor' (associated with biotinylated antibody), following excitation of the donor, if the two were in close proximity as a result of successful binding of biotinylated antibody and Eu-TBP-K-labelled antigen. However if periprep scFv successfully bound the Eu-TBP-K 5T4-labelled antigen, inhibiting the binding of biotinylated ZY07N8-A07 IgG, then FRET was reduced or abolished. Data was analysed as follows:-

i). Ratio = (counts at 665nm / counts at 620nm) x 10,000

ii). Delta F = ((Sample ratio – non-specific mean ratio) / non-specific mean ratio).

iii) Normalized = (Sample Delta F/Mean Total Delta F) x 100

[0396] Definition of hits varied from assay to assay. Where positive control samples gave significant responses, the cut-off was set as the normalized value equal to or greater than the best positive control. Where positive control responses were lower than expected or absent (due presumably to poor expression as scFv) hits were defined as any periprep scFv sample which gave a normalized value that was 3 SD or more beyond the periprep scFv negative control, CEA6.

[0397] Hits from periprep scFv screening were produced as purified hisprep scFv and evaluated for the ability to compete biotinylated ZY07N8-A07 IgG for binding to Eu-TBP-K human and cyno 5T4 ECD Fc, as follows:-

[0398] Each hisprep scFv was tested at 11 concentrations, 25% final top concentration and 10 x 1-in-2 serial dilutions. Since the stock concentration of each hisprep scFv varied, so did the final concentration range for each sample. Hisprep scFv were combined with either a 1 in 408 dilution of Eu-TBP-K human 5T4 ECD Fc, or a 1 in 450 dilution of EU-TBP-K cyno 5T4 ECD Fc in Corning-Costar 384-well assay plates, and pre-incubated for 15 minutes. 0.8nM biotinylated ZY07N8-A07 IgG and 10nM SAXL665 were then added. Total binding wells contained biotinylated ZY07N8-A07 IgG and Eu-TBP-K human or cyno 5T4 ECD Fc, SAXL665 and assay buffer, but no hisprep scFv. Non-specific binding wells contained Eu-TBP-K human or cyno 5T4 ECD Fc, SAXL665 and assay buffer, but no hisprep scFv and no biotinylated ZY07N8-A07 IgG. Total well volume was 20µl with each reagent being added in 5ul. Any shortfall was made up with assay buffer. Assay plates were lidded, covered in foil and incubated at room temperature for 4hrs, before being counted on a Perkin Elmer Envision plate reader using excitation wavelength 320nm and emission wavelengths 620 and 665nm.

[0399] Data was analysed as follows:-

i). Ratio = (counts at 665nm / counts at 620nm) x 10,000

ii). Delta F = ((Sample ratio – non-specific mean ratio) / non-specific mean ratio).

[0400] Any concentration-dependant inhibition of the binding of ZY07N8-A07 IgG to Eu-TBP-K labelled 5T4 ECD Fc by hisprep scFv was plotted using Graphpad Prism software, Log Conc (M) vs Delta F. Curves were fitted using non-linear regression, and IC50 values determined. Figure 7 provides an example of inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K human 5T4 ECD Fc by hisprep scFv using HTRF.

Table 11. IC50 data for example of inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K human 5T4 ECD Fc by hisprep scFv using HTRF

Clone ID	Human 5T4 ECD Fc Binding IC50 (nM)
ZY07N8-A07-GL	302 (n=1)
5T4-60	2.5 (n=1)
5T4-68	11 (n=1)
5T4-69	14 (n=1)

[0401] Figure 8 provides an example of inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K cyno 5T4 ECD Fc by hisprep scFv using HTRF.

Table 12. IC50 data for example of inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K cyno 5T4 ECD Fc by hisprep scFv, using HTRF

Clone ID	Cyno 5T4 ECD Fc Binding IC50 (nM)
ZY07N8-A07-GL	87 (n=1)
5T4-60	0.53 (n=1)
5T4-68	9.5 (n=1)
5T4-69	19 (n=1)

Table 13. Mean IC50 data for inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K human 5T4 ECD Fc by hisprep scFv using HTRF

Clone ID	Human 5T4 ECD Fc Binding Mean IC50 (nM)
ZY07N8-A07-GL	257 +/- 21 (n=5)
5T4-60	1.7 (n=2)
5T4-68	11 (n=1)
5T4-69	14 (n=1)

Table 14. Mean IC50 data for inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K cyno 5T4 ECD Fc by hisprep scFv using HTRF	
Clone ID	Cyno 5T4 ECD Fc Binding Mean IC50 (nM)
ZY07N8-A07-GL	79 +/- 9.6 (n=6)
5T4-60	0.39 (n=2)
5T4-68	9.5 (n=1)
5T4-69	19 (n=1)

Epitope competition assay of re-formatted IgGs

[0402] Purified human IgGs were assessed for their ability to compete biotinylated ZY07N8-A07 IgG for binding to Eu-TBP-K labelled human and cyno 5T4 ECD Fc. Each novel unlabeled IgG was tested at 11 concentrations (neat undiluted sample, plus 10 x 1-in-2 serial dilutions in assay buffer). Since the stock concentration of each IgG varied, the final tested concentration range of each IgG also varied. In Corning-Costar 384-well black assay plates, IgGs were combined with a 1 in 408 dilution of Eu-TBP-K human 5T4 ECD Fc and a 1 in 408 or 1 in 450 dilution of Eu-TBP-K cyno 5T4 ECD Fc, then pre-incubated for 15 minutes, before addition of 0.8nM biotinylated human ZY07N8-A07 IgG and 10nM SAXL665. Total binding wells contained biotinylated A07 IgG and Eu-TBP-K human or cyno 5T4 ECD Fc but no unlabeled competing IgG. Non-specific binding wells contained Eu-TBP-K human or cyno 5T4 ECD Fc and SAXL665 but no biotinylated IgG and no unlabeled IgG. Total assay volume was 20µl/well, with each reagent being added in 5µl. Any shortfall was made up with assay buffer. The plates were lidded, covered in foil and incubated at room temperature for 4hrs before being counted on

a Perkin Elmer Envision plate reader, excitation wavelength 320nm and emission wavelengths 620 and 665nm.

[0403] Data was analysed as follows:-

i). Ratio = (counts at 665nm / counts at 620nm) x 10,000

ii). Delta F = ((Sample ratio – non-specific mean ratio) / non-specific mean ratio).

[0404] Any concentration-dependant inhibition of biotinylated ZY07N8-A07 IgG binding to 5T4 ECD Fc was plotted using Graphpad Prism software, Log Conc. (M) vs. Delta F. Curves were fitted using non-linear regression, and IC50 values determined. Figure 9 provides an example of inhibition of biotinylated ZY07N8-A07 IgG binding to Eu-TBP-K human 5T4 ECD Fc by human IgGs using HTRF.

Table 15. IC50 data for example of inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K human 5T4 ECD Fc by human IgGs using HTRF

Clone ID	Human 5T4 ECD Fc Binding IC50 (nM)
A07-GL	2.2 (n=1)
5T4-60	0.70 (n=1)
5T4-65	0.72 (n=1)
5T4-68	0.64 (n=1)
5T4-69	0.83 (n=1)
5T4-107	0.17 (n=1)
5T4-108	0.70 (n=1)
5T4-112	1.6 (n=1)

[0405] Figure 10 provides an example of inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K cyno 5T4 ECD Fc by human IgGs using HTRF.

Table 16. IC50 Data for example of inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K cyno 5T4 ECD Fc by human IgGs using HTRF	
Clone ID	Cyno 5T4 ECD Fc Binding IC50 (nM)
A07-GL	1.36 (n=1)
5T4-60	0.80 (n=1)
5T4-65	0.47 (n=1)
5T4-68	0.80 (n=1)
5T4-69	1.4 (n=1)
5T4-107	0.53 (n=1)
5T4-108	0.69 (n=1)
5T4-112	1.7 (n=1)

Table 17. Mean IC50 data for inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K human 5T4 ECD Fc by human IgGs, using HTRF	
Clone ID	Human 5T4 ECD Fc Binding Mean IC50 (nM)
A07-GL	2.1 +/- 0.0088 (n=3)
5T4-60	0.70 (n=1)
5T4-65	0.82 (n=2)
5T4-68	0.64 (n=1)
5T4-69	0.83 (n=1)

5T4-107	0.17 (n=1)
5T4-108	0.68 (n=2)
5T4-112	1.6 (n=1)

Table 18. Mean IC50 data for inhibition of biotinylated ZY07N8-A07 IgG binding to EU-TBP-K cyno 5T4 ECD Fc by human IgGs, using HTRF

Clone ID	Cyno 5T4 ECD Fc Binding
	Mean IC50 (nM)
A07-GL	1.2 +/- 0.12 (n=3)
5T4-60	0.80 (n=1)
5T4-65	0.73 (n=2)
5T4-68	0.80 (n=1)
5T4-69	1.4 (n=1)
5T4-107	0.53 (n=1)
5T4-108	0.63 (n=2)
5T4-112	1.7 (n=1)

Example 3 - In vitro characterization of affinity-optimized antibodies

Re-formatting to T289C_A339C IgG, site-specific conjugation with tubulysin and confirmation of improved in vitro cytotoxicity (DU-145 prostate cells)

[0406] The lead scFv were re-formatted to human IgG1 containing the mutations T289C and A339C (based on EU numbering, ref) to allow conjugation of the tubulysin payload at these engineered cysteine residues.

[0407] Conjugation to the engineered cysteines was carried out by first performing by mild reduction by using a 40-fold molar excess of the reducing agent TCEP for 3 hours at room temperatue in PBS. The reducing agent was then removed using desalting columns or dialysis and the antibody interchain disulphide bonds were allowed to re-form by addition of a 20-fold molar excess of dehydroscorbic acid (dhAA) to antibody for 4 hours at room temperature. The maleimide-based tubulysin drug was then added at a 15-fold molar excess to antibody for 16 hours at 4°C to allow conjugation. The reaction was quenched with a 4-fold molar excess of N-acteyl cysteine (NAC) to drug and the resulting conjugated antibody was purified. The unconjugated and conjugated antibodies were analysed by non-reduced and reduced SDS-PAGE to confirm purity and conjugation to the heavy chain (data not shown).

Determination of binding kinetics using Surface Plasmon Resonance (SPR): ADC vs. naked antibody

[0408] The Biacore T-100 instrument was used to characterize binding of the lead candidate ADC 5T4_0108 to monomeric human and cyno 5T4 ECD. To perform kinetic analyses of monomeric human & cyno 5T4 ECD binding, Abs were affinity captured using an anti-human Fc antibody. The binding in this assay format could be described very accurately with the Langmuir 1:1 model, theoretical and experimental Rmax values show very good correlation, and regeneration with 3M MgCl2 was complete. Pilot tests have shown that there was no nonspecific binding of 5T4 ECD to the carboxymethylated dextran matrix of the CM3 chip. Unconjugated 5T4_0108 was also tested to ensure that the conjugation of tubulysin does not interfere with the antigen recognition and binding. Indeed the binding affinities obtained are comparable between the ADC and the unconjugated IgG. The affinity of 5T4_0108 for binding to cynomolgus 5T4 ECD was also tested, with less than 2-fold difference to human 5T4 ECD.

Table 19. Summary of the binding kinetics for 5T4_0108 ADC and IgG. The expRmax stands for experimental Rmax, and thRmax stands for calculated theoretical Rmax based on the molecular weights of the interactants, the stoichiometry of the interaction and the immobilization level of the ligand

ANALYTE : human 5T4 EDC					
LIGANDS	**K_d (nM)**	**k_{on} (M^{-1} s^{-1})**	**k_{off} (s^{-1})**	**R_{max} (exp/th) (RU)**	**Chi^2**
0108 ADC (173 RU)	6.5	3.28×10^5	0.002	94.9 (92.3)	1.44
0108 IgG (118 RU)	6.5	3.3×10^5	0.00213	68.3 (63)	0.75
ANALYTE : cyno 5T4 EDC					
LIGANDS	**K_d (nM)**	**k_{on} (M^{-1} s^{-1})**	**k_{off} (s^{-1})**	**R_{max} (exp/th) (RU)**	**Chi^2**
0108 ADC (173RU)	9.6	1.04×10^5	0.001	82.1 (91.2)	0.5

Specificity/selectivity of 5T4_0108 by ELISA

[0409] The same assay was performed as described in Example 1(H) to check specificity of the affinity optimized lead 5T4_0108. 5T4_0108 was confirmed to be specific for human 5T4 over the closest related human antigens. Figure 11 shows a specificity ELISA 5T4_0108 demonstrating specific binding to human 5T4.

In vitro cytotoxicity of 5T4_0108_FC and FCC tubulysin based ADCs to DU-145 and N87 tumor cell lines

[0410] The lead antibody was also reformatted to 2 alternative human IgG1 formats called FC and FCC containing the following mutations in the heavy chain: L234F and S239C

(FC) and L234F, S239C and S442C (FCC) (based on EU numbering). Conjugation to the tubulysin drug was then carried out as above. Drug loading and purity confirmed by LC-MS.

Example 4 Conjugation of a 5T4-PBD ADC

[0411] 108(FC)-Comp64 is made using site-specific conjugation technology where the PBD payload is conjugated to specific cysteines engineered into the antibody . Initially, the 108(FC) antibody is partially reduced by adding the antibody, at a 5-10mg/ml concentration, to tris(2-carboxyethyl)phosphine (TCEP) dissolved in a pH-adjusted PBS EDTA buffer such that the resulting antibody/TCEP solution had a TCEP/mAb molar ratio of 40. The antibody/TCEP solution was incubated at 37°C for 2.5 – 3 hours. The reduced antibody was buffer exchanged into conjugation reaction buffer (PBS, 1mM EDTA, pH 7.2) using NAP columns for mg scale conjugations or with tangential flow filtration (TFF) for g scale conjugations. Re-oxidation was initiated by the addition of dehydrascorbic acid (dHAA) in DMSO solution to achieve a dhAA/mAb molar ratio of 20, and incubation at 20°C for 3-4 hours. The Comp64 PBD payload is prepared by dissolving it in DMSO to achieve a final concentration of 10mM. For the conjugation reaction, DMSO is added slowly to the de-capped antibody to a final concentration of 10% v/v, and subsequently the payload is added to the de-capped antibody to achieve a payload/mAb molar ratio of 12. The payload/mAb solution is incubated at 20°C for 1 hour and the conjugation reaction is quenched by the addition of N-acetylcysteine (NAC) solution to achieve a NAC/mAb molar ratio of 48 and subsequent incubation at 20°C for 15 minutes. For mg-scale conjugations the PBS conjugation buffer is exchanged against histidine buffer (25mM histidine HCl, 200mM sucrose and 0.02% w/v PS80) using a NAP column. For g-scale conjugations, buffer exchange into 25mM histidine, pH 6 was performed using TFF and final formulation was achieved by adding 25% v/v of conditioning buffer (25mN histidine, 1M

sucrose, .1% PS80, pH 6.0). The final ADC product was formulated at 3mg/ml in formulation buffer (25mN histidine, 0.2M sucrose, 0.02% PS80, pH 6.0).

[0412] The biochemical properties of the resulting ADC are characterized using size-exclusion chromatography high pressure liquid chromatography (SEC-HPLC) to determine purity and aggregation content, and by using hydrophobic interaction chromatography HPLC (HIC-HPLC) to confirm drug loading. Reduced glycosylated reverse-phase HPLC (RP-HPLC) and liquid chromatography-mass spectrometry (LC-MS) were conducted to determine the drug:antibody ratio (DAR) and specificity of site-specific conjugation. Typically these conjugation reactions produced ADCs with >98% monomer, with a conjugation efficiency of >90% correlating to a DAR >1.82.

Example 5 - Binding and Internalization Studies with a 5T4-PBD ADC

Binding Studies

[0413] Human cancer cell lines that express 5T4, the CYNOM-K1 cynomolgus cell line that also expresses 5T4, as well as negative control Daudi and Raji cell lines that lack 5T4 expression were plated at a density of 250,000 cells per well in tissue-culture treated 96-well plates and kept on ice. In studies utilizing a secondary antibody, the 108(FC) or 108(FC)-Comp64 were diluted in DPBS+2% fetal bovine serum (FBS) and were added to the cells at a final concentration of 10μg/ml. The plates were incubated on ice for 30 minutes followed by 2 washes with DPBS+2% FBS, after which, the secondary antibody (Goat anti-human FITC) was added to each well at a final concentration of 15μg/ml. The plates were incubated on ice for an additional 30 minutes followed by an additional 2 washes with DPBS+2% FBS. The cells were resuspended with DPBS+2% FBS plus DAPI at a concentration of 3 μM and the mean fluorescence intensity was measured using a flow cytometer. In studies where the antibody or

ADC itself was labelled, the molecule was labelled with the AF647 fluorophore, and the labelled molecule was added to the plates at a concentration of 0.5µg/ml. The plates were then washed as described above and the cells were collected and analyzed using a flow cytometer.

[0414] Figure 12 and Table 20 demonstrate that a number of human tumor cell lines representing various indications do have significant 5T4 expression on the cell surface as indicated by binding to the various candidate parental anti-5T4 antibodies. These will therefore provide a number of options for testing 108(FC)-Comp64 in in vitro and in vivo experiments. In addition, the parental candidate antibodies demonstrate cross-reactivity with cynomolgus 5T4 which is crucial for downstream toxicology studies (Figure 13).

Table 20. Fluorescence Intensity

5T4 Ab	DU-145	PC3	Calu-1	NCI-N87	786-O	SKOV3	HCT116
Isotype Ctl	1	1	1	1	1	1	1
A07	4.5	1.9	4.2	1.9	3.9	1.5	2.0
G01	4.3	2.2	5.4	1.4	4.5	1.7	2.6
D04	2.8	1.4	3.0	1.2	2.7	1.2	1.9
A03	2.6	2.1	5.4	2.0	4.6	1.6	2.5
C04	2.9	1.5	3.0	1.3	3.0	1.6	1.6

*Fluorescence Intensity=GeoMean of anti-5T4 Ab divided by GeoMean of isotype control Ab

Internalization of Candidate Parental anti-5T4 Antibodies

[0415] A panel of human cancer cell lines that express various levels of 5T4 (DU 145, PC3, HCT-116, NCI-N87, Calu-1, 786-0 and SKOV3) and negative control cells that lack 5T4 expression (Daudi and Raji) were used to determine the relative internalization rates of the

candidate parental anti-5T4 antibodies by flow cytometry. Unlabeled candidate anti-5T4 antibodies were incubated with 20,000 at a concentration of 5μg/ml. For binding controls, one set of cells were kept on ice for 2 hours. For internalization experiments, another set of cells were incubated at 37°C to allow for internalization. At two time points, 20 minutes or 2 hours, the cells incubated at 37°C were washed with ice cold PBS buffer and then incubated with a fluorescently-labeled anti-human IgG secondary antibody at 1 μg/ml concentration for 30 minutes on ice. After 2 washes with PBS, the cells were harvested and analyzed by flow cytometry to calculate mean fluorescent intensity. The amount of anti-5T4 antibody internalized into cells at each time point was determined by the percentage decrease of mean fluorescence intensity as compared to control cells that were incubated at 4C for the same period of time.

[0416] Figure 14 and Table 21 demonstrate that the candidate parental anti-5T4 internalize well into most cancer cell lines within 2 hours. In Table 21, the % bound Ab internalized is equal to the amount of anti-5T4 antibody internalized into cells after incubation at 37°C for 2 hours were determined by the percentage decrease of mean fluorescence intensity as compared to control cells that were incubated at 4°C for 2 hours.

[0417] To confirm the internalization of these parental antibodies and to predict if they can deliver a cytotoxic payload, the parental antibodies were tested in the Hum-Zap assay from Advanced Targeting Systems according to manufacturer's instructions. Briefly, 5T4-positive DU 145 cells were plated in culture media at a density of 2,000 per well of tissue culture-treated 96-well plates and allowed to adhere overnight at 37°C/5% CO_2. To prepare test articles, each parental antibody was incubated with a secondary antibody (goat anti-human IgG) conjugated with the ribosome inactivating protein, saporin, for 30 minutes at room temperature to form a secondary conjugate. Serial dilutions of this secondary conjugate were then prepared and added

to cells. Following incubation at 37°C/5% CO_2 for 72 hours, the CellTiter-Glo Luminescent Viability Assay from Promega was used to determine relative cytotoxicity. Briefly, CellTiter-Glo reagent was added to each well, allowed to incubate for 10 minutes at room temperature with mild shaking and then the absorbance of each sample at 560nM was read using a Perkin Elmer EnVision luminometer. The percent cell viability was calculated by the following formula: (average luminescence of treated samples/average luminescence of control samples) x 100. IC_{50} values were determined using logistic non-linear regression analysis with GraphPad Prism software.

[0418] Figure 14B and Table 21B demonstrate that the candidate parental anti-5T4 antibodies are able to internalize and deliver the saporin-conjugated secondary leading to potent cytotoxicity of the 5T4 positive DU 145 cells. In Table 21B, all the 7 leads have tumor cell-killing activity in saporin-conjugated cytotoxicity assay (ZAP assay). Five Leads and two benchmark antibodies were then conjugated to an auristatin microtubule inhibitor (DARs ~4-5).

Table 21A. Internalization of parental anti-5T4 antibodies by DU 145 human prostate cancer cells (showing % of bound antibody)

Tumor cell type	D04	C04	G01	A07	A03
DU-145 prostate	93	83	53	66	56
PC3 prostate	84	79	28	51	40
Calu-1 lung	84	70	30	36	18
NCI-N87 gastric	93	95	81	48	41
786-O RCC	57	47	36	37	23
SKOV3 ovarian	69	63	54	7	41

HCT-116 colon	65	68	46	46	4

Table 21B. Parental anti-5T4 antibodies tested in the Hum-Zap saporin-conjugate assay induce cytotoxicity of 5T4-positive DU 145 prostate cancer cells

5T4 Antibody	IC50 (nM)
ZY07N1-G01	0.40
ZY07PH-C04	0.66
ZY07N7-A03	0.67
ZY02Q3-C08	0.62
ZY07N8-A07	0.70
ZY07NG-D04	0.64
ZY07NB-F02	2.5

Internalization Kinetics of Lead Anti-5T4 Antibodies and Conjugates

[0419] Human prostate cancer cell lines DU145 was maintained in DMEM medium with 10% FBS. On the day of assay, the cells were detached from the flask with 0.25% trypsin and washed twice with ice cold PBS. The cells were plated into 96-well U-bottom plates at a density of 100,000 cells/well. AlexaFluor-488-conjugated parental A07 antibody, the affinity-optimized 108(FC) antibody, and a tubulysin conjugate with the affinity-optimized antibody (108{FC}-1508 were diluted in binding medium (DMEM, 3%BSA and 20mM HEPES) at 2 µg/ml and were added into each of the corresponding wells containing cells at 50 µl/well. After incubation on ice for 1 h, the antibodies were removed by pelleting the cells at 1500 rpm for 5 min at 4°C

and discarding the supernatant. The cells were washed twice with ice cold PBS, and 100 µl of fresh binding medium was added to each well. The plates were incubated either on ice (time 0) or at 37°C/5% CO_2 for 10, 20, 30, 60 or 120 minutes. At the end of each incubation, the cells were washed twice with ice-cold PBS 2X and the cells were incubated on ice. A quenching antibody, Anti-AlexaFluor-488, was added to the cells at a final concentration of 25 µg/ml and the cells were incubated on ice for 30 minutes to quench the signals of antibodies that were not internalized. The cells were washed twice with ice-cold PBS and then analyzed via flow cytometry using an LSRII instrument. The data was normalized by subtracting background and unquenched signals. The internalization was displayed as the percentage of unquenchable signal (i.e. the signal from internalized molecules) from cells incubated at 37°C for the various time points per the signal from cells at time 0.

[0420] Figure 15 demonstrates that the lead antibody, 108(FC) and a tubulysin conjugate with this antibody each have efficient and comparable internalization kinetics where ~70% of the antibody is internalized within two hours compared to 66% for parental A07 antibody. Conjugation of a tubulysin to 108(FC) does not affect the internalization rate.

Lysosomal Localization Studies

[0421] DU 145 human prostate cancer cells, which express 5T4, were transfected to overexpress the lysosomal marker LAMP-1 that is tagged with an mCherry fluorescent label (DU 145-LAMP-1). DU 145-LAMP-1 cells were plated at a density of 50,000 cells per well in 8-well chamber slides and kept on ice. The 108(FC) antibodies that were fluorescently conjugated with AlexaFluor-488108(FC) were diluted in PBS + 2% FBS and added to the cells at a final concentration of 20-40µg/ml. After incubating at 37°C for 1 hour, the cells were fixed

in 200µl of 3.7% paraformaldehyde, washed twice with PBS + 2% FBS and imaged with confocal microscopy to look for colocalization of the antibody with LAMP-1.

[0422] Figure 16 demonstrates that the 108(FC) antibody binds to the surface of DU 145-LAMP-1 cells, is internalized, and colocalizes to the lysosomes as indicated by the co-localization of fluorescent signals.

Example 6 - In Vitro Cytotoxicity Assay with a 5T4-PBD ADC

Inhibition of Cancer Cell Growth

[0423] A panel of human cancer cell lines that express various levels of 5T4 (DU 145, NCI-N87, MDA-MB-361, H1975) and negative control cells that lack 5T4 expression (Raji and SNU-16) were used to determine the relative cytotoxicity of the lead antibody, 108(FC) or ADCs composed of the lead antibody conjugated to various payloads including an undisclosed tubulysin payload and various PBD payloads. The cells were plated in culture media at a density of 2,000 to 5,000 per well (depending on the growth kinetics of each cell line) of tissue culture-treated 96-well plates in a volume of 80µl and allowed to adhere overnight. A 5X concentration of each dose of antibody or ADC to be tested was prepared by diluting the test articles in culture medium. Twenty microliters of each test article was added to cells in duplicate or triplicate such that the final dose curve ranged from 4µg/ml down to 61pg/ml in a stepwise 1:4 serial dilution series. The treated cells were cultured at 37°C / 5% CO_2 for 72 to 144 hours (depending on the growth kinetics of each particular cell line). The CellTiter-Glo Luminescent Viability Assay from Promega was used to determine relative cytotoxicity. Briefly, 100µl of CellTiter-Glo reagent was added to each well, allowed to incubate for 10 minutes at room temperature with mild shaking and then the absorbance of each sample at 560nM was read using a Perkin Elmer EnVision luminometer. The percent cell viability was calculated by the following formula:

(average luminescence of treated samples/average luminescence of control samples) x 100. IC_{50} values were determined using logistic non-linear regression analysis with GraphPad Prism software.

[0424] Unconjugated 108(FC) had no effect on the growth of any of the cell lines tested (data not shown). The affinity optimized antibody 108(FC) had significantly increased potency as an ADC compared to the parental A07 antibody (Figure 17 and Table 22). Table 22 shows that affinity-optimized ADCs are significantly more potent than parentals against DU 145 human prostate cancer cells.

Table 22. Summary of *In Vitro* cytotoxicity vs. KD

ADC (Max Conc. 4ug/ml) T289C-A339C + Tubulysin (DAR ~4)	*In vitro* cytotoxicity (DU 145)			KD determination (BIAcore)		
	IC50 (ng/ml)	Max Kill (%)	Fold vs. parent	Human 5T4 (nM)	Cyno 5T4 (nM)	Fold vs. parent
A07 (parent)	140.9	64		94	nd	
108 (A07 variant)	1.5	74	94	6.5	9.1	14

[0425] 108(FC)-based ADCs very potently inhibited the growth of a variety of 5T4-positive tumor cells (Figures 18A-B (representative assay), Table 23).

Table 23. Average IC_{50} values for 108(FC) and conjugates incorporating the 108(FC) antibody

	IC_{50} Cytotoxicity Values (ng/ml)			
	DU 145	NCI-N87	MDA-MB-361	H1975
108(FC)	NA	NA	NA	NA
108(FC)-1508	2.5	10	0.4	~148
108(FC)-Auristatin	6.0	25	NT	NT
108(FC)-Comp64	2.2	~100	0.2	~800
108(FC)-Comp18	2.2	NT	NT	NT
108(FC)-Comp96	NT	NT	0.3	NT

[0426] While 108(FC)-1508 potently inhibited the growth of 5T4-positive tumor cells (Figure 19A), 108(FC)-Comp64 had either comparable (Figure 19A) or superior (Figure19B) cytotoxicity to the 108(FC)-1508 ADC, depending on the cell line. Additionally, 108(FC) conjugated to other PBD dimer payloads such as Comp96. Conjugating the tubulysin payload to the 108(239iCys) antibody resulted in potent cytotoxicity against 5T4-positive MDA-MB-361 cells (Figure 20). Neither 108(FC) nor any of the ADCs tested had any effect on the growth of 5T4-negative cells, indicating target-specific cytotoxicity (data not shown).

Bystander-kill activity of 108(FC)-Comp64 and 108(FC)-Comp96

[0427] ADCs composed of the 108(FC) conjugated with either of the PBD payloads, Comp64 or Comp96 were evaluated for the ability to eradicate 5T4-negative cancer cells in a mixed cell population of both 5T4-positive and 5T4-negative cells through bystander-kill activity. MDA-MB-361 breast cancer cells with high 5T4 expression and DMS114 lung

carcinoma cells with extremely low/negative 5T4 expression were used in these studies. Cells were plated in 80 µL of RPMI1640 with 10% FBS into 96-well flat-bottomed plates according to the following conditions: 2,000 MDA-MB-361 cells/well, 2,000 DMS114 cells/well, or 1,000 MDA-MB-361 + 1,000 DMS114 cells/well. Cells were allowed to adhere overnight. A 5X concentration of each ADC was prepared by diluting the test articles in culture medium. Twenty microliters of each test article was added to cells in triplicate such that the final dose curve range of 50 ng/mL down to 0.76 pg/mL in a stepwise 1:4 serial dilution series was tested against 5T4-positive, 5T4-negative or mixed-cell populations. The treated cells were cultured at 37°C/5% CO2 for 6 days and cell viability was assessed with the CellTiter-Glo Luminescent Viability Assay from Promega. 100 µL of reconstituted CTG reagent was added each well, mildly shaken for 10 minutes at room temperature, and the absorbance of each sample at 560nM was read using a Perkin Elmer EnVision luminometer. The percent cell viability was calculated by the following formula: (average luminescence of treated samples/average luminescence of untreated control samples) x 100. IC_{50} values were determined using logistic non-linear regression analysis with GraphPad Prism software. Bystander-kill activity is defined as when the ADC is able to kill not only antigen-positive tumor cells but also proximally-located antigen-negative tumor cells.

[0428] The data in Figures 21 and 22 clearly demonstrate that 108(FC)-Comp64 and 108(FC)-Comp96, respectively, are equally capable of inducing bystander-kill activity against 5T4-negative DMS114 lung carcinoma cells when these are co-cultured with 5T4-postiive MDA-MB-361 cells. However, neither is capable of inducing cytotoxicity of DMS114 cells when these are cultured alone.

Example 7 - *In Vivo* Efficacy Xenograft Studies

Cell-based Xenograft Studies

[0429] Athymic (nude) mice were subcutaneously injected with 500,000 cells (diluted in PBS + Matrigel in a 1:1 ratio) from various human tumor cell lines (DU 145, NCI-N87, MDA-MB-361, H1975) and tumors were allowed to develop. The mice were size-matched based on tumor volume and assigned to treatment groups such that each treatment group contained 8-10 mice with an average tumor volume of 200mm^3 for each cohort.

[0430] Test articles for various studies, which were administered intravenously, included vehicle (formulation buffer), 108(FC)-1508, 108(FC)-Comp64 and a non-targeting control antibody conjugated with the Comp64 payload, NC(FC)-Comp64. An Antibody-Drug mix containing unconjugated antibody and free warhead at levels comparable to the highest dose of an ADC tested were also included as a control. The dosing regimens tested doses ranging from 0.1mg/kg up to 5mg/kg where these were administered either as a single dose, QWKx3, QWKx5, or Q3WKx3. Tumors were measured twice a week throughout the course of the study and the following formula was used to calculate tumor volume: (length x width^2)/2 = mm^3.

[0431] The data in Figure 23 demonstrates that 108(FC)-1508 administered QWKx5 is able to significantly inhibit the growth of DU 145 xenografts in a dose-dependent manner, with initial regression with subsequent regrowth. In contrast to in vitro data that demonstrated similar efficacy with 108(FC)-1508 and 108(FC)-Comp64 in this cell line (Figure 19A and B), significantly more potent and more durable responses are observed in vivo with 108(FC)-Comp64 against DU 145 xenografts. Durable tumor regression is observed following treatment with this ADC at 1mg/kg QWKx5 (Figure 23). In this same model, 108(FC) conjugated to a different PBD dimer payload, Comp18, demonstrated similar efficacy at the doses tested (Figure 24).

[0432] The data in Figures 25A and 25B demonstrate that a single administration of 108(FC)-Comp64 was able to either inhibit the growth of or regress NCI-N87 gastric carcinoma xenografts in a dose-dependent manner. The most efficacious treatment was a single administration of a 1mg/kg dose which resulted in long-term regressions. Alternative dosing regimens were tested in this model and administering 108(FC)-Comp64 at a dose of 0.33 mg/kg on either a QWk x 3 or a Q3Wk x 3 regimen resulted in comparable regression with slightly better efficacy observed with the more frequent schedule.

[0433] The data in Figures 25C and 25D demonstrate that a single administration of 108(FC)-Comp64 was able to either inhibit the growth of or regress MDA-MB-361 breast cancer xenografts in a dose-dependent manner. The most efficacious treatment was a single administration of a 1mg/kg dose which resulted in long-term regressions out to 60+ days post-treatment. Alternative dosing regimens were tested in this model and administering 108(FC)-Comp64 at a dose of 0.33 mg/kg on either a QWk x 3 or a Q3Wk x 3 regimen resulted in comparable regression with slightly better efficacy observed with the more frequent schedule. Though the response to these regimens was slightly delayed compared to the 1mg/kg dose, all three regimens resulted in nearly complete regression of tumors by the end of the study (Figure 25D).

[0434] The data in Figures 26A and 26B demonstrate that a single administration of 108(FC)-Comp64 was able to either inhibit the growth of or regress MDA-MB-361 breast cancer xenografts in a dose-dependent manner. The most efficacious treatment was a single administration of a 1mg/kg dose which resulted in long-term regressions out to 80+ days post-treatment. Alternative dosing regimens were tested in this model and administering 108(FC)-Comp64 at a dose of 0.33 mg/kg on a QWk x 3 regimen resulted in similar efficacy to the single

administration of the 1mg/kg dose (Figure 26B). In this model administering 108(FC)-Comp64 on a Q3Wk x 3 basis resulted in tumor growth inhibition with no significant regression observed in this treatment group (Figure 26B).

Human gastric cancer patient-derived xenograft studies

[0435] Each of the human gastric cancer patient-derived xenograft (PDX) models was established from a surgically-resected clinical tumor sample, and implanted into nude mice defined as passage 0 (P0). When tumors developed they were passaged from these donor mice into recipient mice defined where this passage was defined as passage 1 (P1) and so on during continual in vivo passaging in mice. Tumors from P3-P6 mice were used in each of the PDX efficacy studies. Certain models were identified that represented various degrees of 5T4 expression: high/homogenous (Models ST-02-0068 and ST-02-0177), low/homogenous (Model ST-02-0164) or high/heterogeneous (Model ST-02-0009) expression as determined by IHC.

[0436] To initiate the efficacy studies, fragments (20-30mm^3) of tumors from P3-P6 mice, were subcutaneously implanted into the right flank of recipient mice for tumor development. Tumor-bearing mice were size-matched into treatment groups of 10 mice each such that the average tumor volume of each cohort was 250mm^3, and treatments were initiated.

[0437] Test articles for various studies, which were administered intravenously, included vehicle (formulation buffer), 108(FC)-Comp64 and a non-targeting control antibody conjugated with the Comp64 payload, NC(FC)-Comp64. The dosing regimens tested a single bolus dose versus a fractionated dosing schedule. Mice were treated with either a single administration of 1mg/kg or received a dose of 0.33mg/kg administered QWKx3. Tumors were measured twice a week throughout the course of the study and the following formula was used to calculate tumor volume: (length x $width^2$)/2 = mm^3.

[0438] The data in Figures 27A and 27B demonstrate that 108(FC)-Comp64 was able to either regress or inhibit the growth of gastric carcinomas in PDX models. In a model with high and heterogeneous 5T4 expression (as determined by IHC), comparable efficacy, i.e. regressions, were observed with either a single administration of 108(FC)-Comp64 at a 1mg/kg dose or a "fractionated" dose of 0.33mg/kg administered QWk x 3 (Figure 27A). In another gastric PDX model with low but homogenous 5T4 expression (as determined by IHC), each treatment again had comparable efficacy but in this model treatment only resulted in significant tumor growth inhibition and no regresssions were observed, likely due to the lower 5T4 expression in this model (Figure 27B).

Example 8 - Activity of Against Cancer Stem Cells

Expression of 5T4 on cancer stem cells

[0439] Cell lines of various different cancer types were tested for 5T4 expression on both total tumor cells as well as CSCs. CSCs were defined as $CD44^+CD24^-$ cells in breast and prostate cancer, $CD90^+$ cells in hepatocellular carcinoma (HCC), and $CD24^+CD44^+$ cells in pancreatic cancer. For staining, the 108(FC) antibody was conjugated to AlexaFluor-750 using the Alexa 750 Microscale Protein Labeling Kit by following manufacturer's instructions. Cells were counted using a Vi-Cell XR Cell Viability Analyzer and resuspended at a density of 5×10^6 cells/mL in ice-cold FACS buffer (Hank's Balanced Salt Solution {HBSS} containing 2% heat-inactivated FBS). One hundred microliters was transferred to appropriate wells in a round bottom, tissue-culture treated 96-well plate and maintained on wet ice for the rest of the protocol. For breast, prostate and pancreatic cancer cell lines the cells were stained with 5 µL of fluorescein conjugated CD24, 5 µL of allophycocyanin-conjugated CD44 and 0.5 µL of AlexaFluor 750-conjugated 108(FC). For HCC, cells were stained with 5 µL PerCP-Cy5.5-

conjugated CD90 and 0.5 µL of AlexaFluor-750-conjugated 108(FC). Cells mixed with appropriate antibodies were incubated on ice for 30 minutes, washed twice with FACS buffer and then run on an LSRII flow cytometer and analyzed with FlowJo software.

[0440] 5T4 was expressed at differing levels on a broad range of cancer cells, with DU 145, SUM159 and Panc-1 showing the highest levels . In all cases, there was an increase of 5T4 surface protein on the CSCs found within these cell lines even when the cell line had little to no 5T4 expression as a whole.

Effects of WARHEAD OF COMP64 and 108(FC)-Comp64 on CSC sphere formation in vitro

[0441] To test the effects of the PBD dimer warhead of Comp64, which is contained in the Comp64 payload, and 108(FC)-Comp64 on CSC sphere formation, cells were plated at a final density of 3000 cells/well in ultra-low attachment plates in DMEM/F12 medium supplemented with 20 ng/mL EGF, 10 ng/mL bFGF, 5 µg/mL insulin and 0.4% bovine serum albumin (BSA). The warhead of Comp64 was added in a range from 4000 to 0.001 nM and 108(FC)-Comp64 was added from 1000-0.004 ng/mL. Cells were cultured at 37°C in a humidified incubator with 5% CO_2 for 4 days. At the end of the incubation period cell viability was measured using CellTiter-Glo Cell Viability Assay following manufacturer's instructions.

[0442] The warhead of Comp64 potently inhibited CSC sphere formation in all lines tested with an IC_{50} range of 0.18-0.78 nM (Figure 28). 108(FC)-Comp64 inhibited both DU 145 (Figure 29) and Sum159 cells (Figure 30) with an IC_{50} of 3.2 and 1.1 ng/ml, respectively. PBD warhed of Comp64 effectively inhibits sphere formation in the sub nM range of all cell lines tested. Determination of the CSC percentages following dosing with 108(FC)-Comp64 in xenograft cancer models

[0443] Tumors from MDA-MB-361 and NCI-N87 xenograft efficacy studies were excised, cut into 4 mm pieces and cryopreserved using Cryostor. Frozen tumor pieces were thawed at 37°C, washed twice in HBSS and further minced using sterile scalpel blades. To obtain single-cell suspensions, the tumor pieces were then mixed with 200 units/mL of ultrapure collagenase III in DMEM/F12 medium. The tumor suspension was incubated at 37°C for approximately 1 hour, with mechanical disruption every 30 minutes by pipetting with a 5-mL pipette. At the end of the incubation, cells were filtered through a 70-µm nylon mesh centrifuged at 1200 rpm for 5 minutes. Cells were washed twice with HBSS. Following the last wash, cells were put through a 40-µm cell strainer and counted using a Vi-Cell XR Cell Viability Analyzer. Cells were assayed for aldehyde dehydrogenase activity as a measure of CSCs using the Stemcell Technologies Aldefluor kit and following manufacturer's instructions. The cells were run on an LSRII flow cytometer and analyzed with FlowJo software.

[0444] There was an approximate 50% reduction in the number of CSCs (Aldfluor+ cells) in both models following treatment with 108(FC)-Comp64 as compared to the control NC(FC)-Comp64 (Figure 31).

Example 9 Transient Expression Analysis for 5T4-108-239iCys

[0445] The 5T4-108-239iCys was transiently transfected into 293F cells using 293fectin™ (Invitrogen cat: 12347-019) using manufacturer's protocol. The cells were cultured in FreeStyle™ 293 Expression Medium (Invitrogen Cat. No. 12338-018) and the culture volume was doubled on day three and day six post transfection. The transfected cells were culture for a total of eleven days. The 5T4-108-239iCys mAb expression level was determined by protein A binding method using A280. The 5T4-239iCys mAb has an expression level that is very similar to the 5T4-WT mAb. The transient expression at day 11 in 293 cells was 204 mg/L.

LCMS drug to antibody determination for 5T4-108-239iCys-1508

[0446] The conjugation was carried out at 1 mg/mL using Medimmune's site-specific conjugation protocol. The efficiency of conjugation was determined by Liquid Chromatography–Mass Spectrometry (LCMS) analysis of the reduced glycosylated conjugated mAb. The relative ratio of peak height intensity was used to calculate the percent of conjugation and the drug to antibody ratio (D.A.R) by comparing conjugated vs. unconjugated species. There was no detectable light chain conjugation suggesting that the conjugation was specific to the heavy chain. The unconjugated heavy chain was 1%, as determined by the ratio of the peak height intensity of the conjugated vs. unconjugated heavy chains. The resulting conjugation efficiency and D.A.R was 99% and 1.98, respectively, as shown in Table 24.

Table 24. D.A.R. Determined by reduced LCMS

Efficiency of conjugation at heavy chain	99%
Non-conjugated antibody	1%
Calculated D.A.R.	1.98

Isoelectric point determination for 5T4-108-239iCys and 5T4-108-239iCys-1508

[0447] The isoelectric point was determined using the iCE280 IEF analyzer following Medimmune's protocol. The analysis was done using carrier ampholytes 8.5 to 10 and pI marker of 9.22 to 10.45. The pI are similar for both the unconjugated and tubulysin conjugated 5T4-108-239iCys, as shown in Table 25 and Figures 32A-B.

Table 25. Isoelectric point

5T4-108-239iCys	9.76
5T4-108-239iCys-1508	9.8

Differential Scanning Calorimetry Analysis

[0448] The lowest transition temperature for 5T4-108-239iCys and 5T4-108-239iCys-1508 was determined using Differential Scanning Calorimetry (VP-DSC). The proteins were extensively dialyzed into 25 mM Histidine buffer pH 6.0 and formulated at 1 mg/mL. After degassing, the proteins were analyzed from 20°C to 100°C at one degree per min raise with a 16 sec filtering period. There was no difference between the conjugated and unconjugated 5T4-108-239iCys transition temperatures. However, the 5T4-WT mAb has a 5°C higher melting temperate for the first transition point. Results are shown in Table 25 and Figures 33A-B.

Table 26. DSC 5T4-108-239iCys-1508	
Lowest TM	62.76°C

Size exclusion chromatography analysis 5T4-108-239iCys and 5T4-108-239iCys-1508

[0449] Size exclusion chromatography (SEC) analysis was done for both the naked mAb and the conjugated mAb with tubulysin. 250 µg of the unconjugated mAb and 100 µg of the conjugated ADC were analyzed using a Tosoh SEC column with 6.0mm ID x 4.0 cm, 7 mm dimension (Cat 08541). The mobile phase consists of 0.1mM sodium phosphate, 0.1mM sodium sulfate pH 6.8 at 1 mL per minute flow rate. Both the conjugated and non-conjugated 5T4-108-239iCyss have high monomeric content. Results are shown in Figure 34.

Hydrophobic Interaction Chromatography 5T4-108-239iCys and 5T4-108-239iCys-1508

[0450] The hydrophobic interaction chromatography (HIC) analysis provides a qualitative look at the ADC in comparison to the naked mAb. The assay was performed using a Tosoh Biosciences TSKgel Butyl-NPR 4.6 mm ID x 3.5 cm, 2.5 mm column (Cat: 14947) using an isocratic gradient. 100 µg of protein was loaded unto the column and the gradient was initiated at 95% mobile phase A (25mM Tris-HCL, 1.5M Ammonium Sulfate, pH 7.0) and concluded at 5% mobile phase B (25mM Tris-HCL, 5% Isopropanol, pH 7.0) in 13 minutes at 1mL per min flow rate. The results indicated that the overall hydrophobicity of the ADC increase with respect to the naked mAb indicating a presence of hydrophobic payload attachment to ADC. Results are shown in Figure 35.

Reduced glycosylated reverse phase analysis of 5T4-108-239iCys and 5T4-108-239iCys-1508

[0451] The reduced glycosylated RP-HPLC was performed using Agilent column RLRP-S, 1000A, 8 mm, 50X2.1 mm (Cat:PL1912-1802). 10 µg of reduced naked mAb and reduced ADC were analyzed using an isocratic gradient from 75% mobile phase A (0.1 % Trifluoroacetic acid) to 25% mobile phase B (0.09 % Trifluoroacetic acid in acetonitrile) over 25 minutes at 80C. The results showed an increase in the retention time (i.e., change of charge) of the heavy chain and not the light chain indicating that the payload is specifically attached to the heavy chain. The conjugated heavy chain has one predominant peak, which suggests conjugation on mainly one payload per heavy chain. Results are shown in Figure 36.

Non-GLP tox buffer stability assessment including 3X F/T

[0452] The stability analysis of the 5T4-108-239iCys-1508 was performed in non-GLP tox buffer (25mM histidine, 7% sucrose, 0.02% polysorbate 80, pH 6.0) using SEC-HPLC and LCMS-D.A.R. analysis. The 5T4-108-239iCys-1508 was subjected to three cycles of freezing at

-80°C and thawing at room temperature. The freeze/thaw material was then incubated at 4°C, 25°C and 40°C for one week. SEC-HPLC analysis were performed on aliquots of the initial material, the 3X freeze/thawed, day 3 and day 7 aliquots, post incubation. The drug to antibody ratio was determined for the initial material and seven days incubated samples. The results indicate that the 5T4-108-239iCys-1508 is stable with minimal loss due to aggregation, fragmentation or deconjugation. Results are shown in Figure 37.

Mouse serum stability of 5T4-108-239iCys-1508

[0453] Mouse serum stability analysis was carried out by incubating 66 µl @at 3 mg/ml of the 5T4-108-239iCys-1508 in mouse serum for zero, three and seven days at 37°C. Immunocapture of 100 µl of the serum incubated ADC was performed using anti-human IgG agarose and the captured ADC after being eluted from the anti-human-Fc was analyzed by reduced LCMS for D.A.R determination. The data show that 5T4-108-239iCys-1508 is stable upon serum incubation; which means there is minimal deconjugation and non-detectable degradation products. Results are shown in Figure 38.

Example 10

General Experimental Methods

[0454] Optical rotations were measured on an ADP 220 polarimeter (Bellingham Stanley Ltd.) and concentrations (*c*) are given in g/100mL. Melting points were measured using a digital melting point apparatus (Electrothermal). IR spectra were recorded on a Perkin-Elmer Spectrum 1000 FT IR Spectrometer. ^{1}H and ^{13}C NMR spectra were acquired at 300 K using a Bruker Avance NMR spectrometer at 400 and 100 MHz, respectively. Chemical shifts are reported relative to TMS (δ = 0.0 ppm), and signals are designated as s (singlet), d (doublet), t (triplet), dt (double triplet), dd (doublet of doublets), ddd (double doublet of doublets) or m (multiplet), with

coupling constants given in Hertz (Hz). Mass spectroscopy (MS) data were collected using a Waters Micromass ZQ instrument coupled to a Waters 2695 HPLC with a Waters 2996 PDA. Waters Micromass ZQ parameters used were: Capillary (kV), 3.38; Cone (V), 35; Extractor (V), 3.0; Source temperature (°C), 100; Desolvation Temperature (°C), 200; Cone flow rate (L/h), 50; De-solvation flow rate (L/h), 250. High-resolution mass spectroscopy (HRMS) data were recorded on a Waters Micromass QTOF Global in positive W-mode using metal-coated borosilicate glass tips to introduce the samples into the instrument. Thin Layer Chromatography (TLC) was performed on silica gel aluminium plates (Merck 60, F_{254}), and flash chromatography utilised silica gel (Merck 60, 230-400 mesh ASTM). Except for the HOBt (NovaBiochem) and solid-supported reagents (Argonaut), all other chemicals and solvents were purchased from Sigma-Aldrich and were used as supplied without further purification. Anhydrous solvents were prepared by distillation under a dry nitrogen atmosphere in the presence of an appropriate drying agent, and were stored over 4Å molecular sieves or sodium wire. Petroleum ether refers to the fraction boiling at 40-60°C.

General LC/MS conditions:

Method 1 (default method, used unless stated otherwise)

[0455] The HPLC (Waters Alliance 2695) was run using a mobile phase of water (A) (formic acid 0.1%) and acetonitrile (B) (formic acid 0.1%). Gradient: initial composition 5% B held over 1.0 min, then increase from 5% B to 95% B over a 3 min period. The composition was held for 0.1 min at 95% B, then returned to 5% B in 0.03 minutes and hold there for 0.87 min. Total gradient run time equals 5 minutes.

Method 2

[0456] The HPLC (Waters Alliance 2695) was run using a mobile phase of water (A) (formic acid 0.1%) and acetonitrile (B) (formic acid 0.1%). Gradient: initial composition 5% B held over 1.0 minute, then increase from 5% B to 95% B over a 2.5 minute period. The composition was held for 0.5 minutes at 95% B, then returned to 5% B in 0.1 minutes and hold there for 0.9 min. Total gradient run time equals 5 minutes.

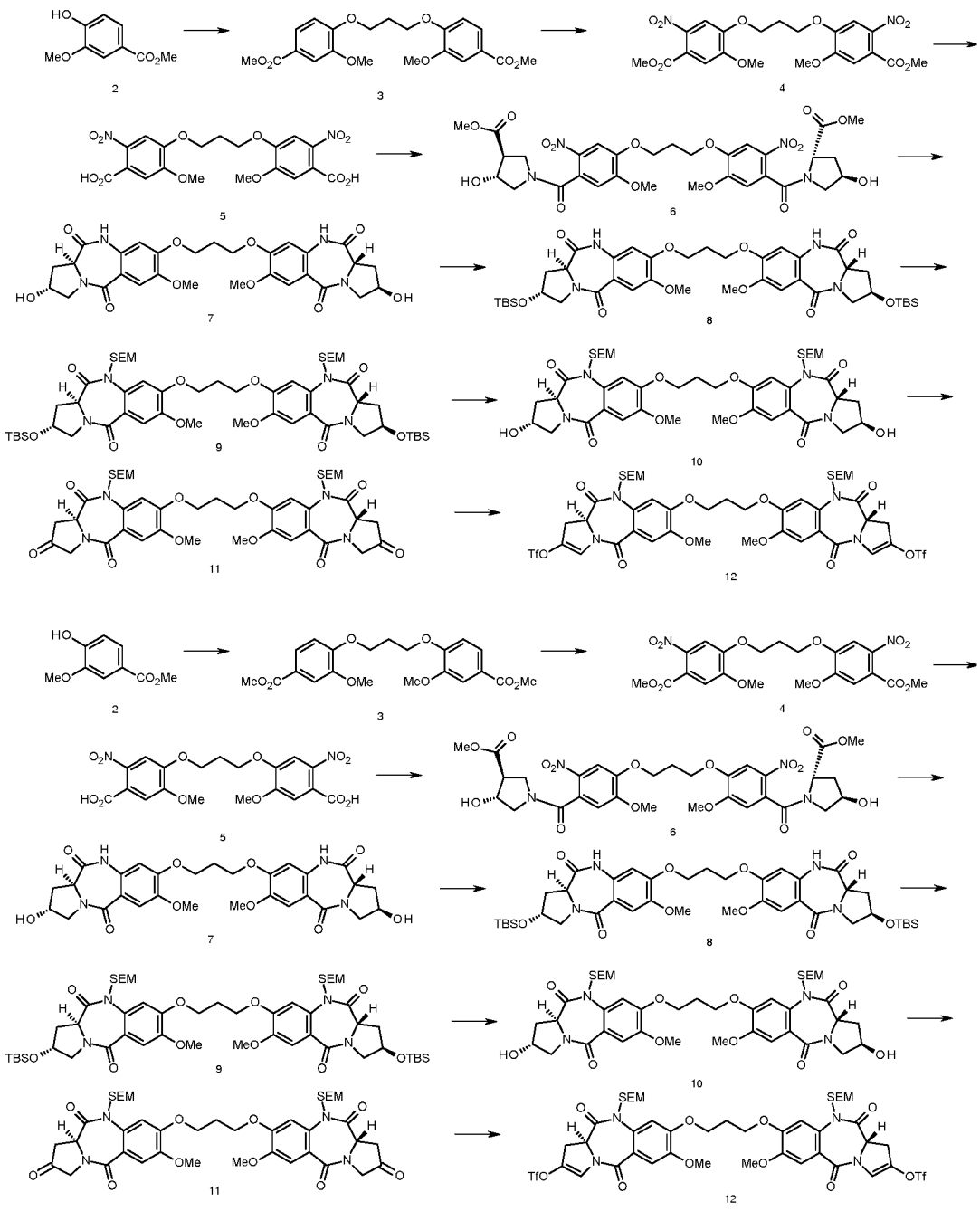
For both methods

[0457] Flow rate 3.0 mL/min, 400µL was split *via* a zero dead volume tee piece which passes into the mass spectrometer. Wavelength detection range: 220 to 400 nm. Function type: diode array (535 scans). Column: Phenomenex Onyx Monolithic C18 50 x 4.60 mm.

[0458] The reverse phase flash purification conditions were as follows: The Flash purification system (Varian 971-Fp) was run using a mobile phase of water (A) and acetonitrile (B). Gradient: initial composition 5% B over 20 C.V. (Column Volume) then 5% B to 70% B within 60 C.V. The composition was held for 15 C.V. at 95% B, and then returned to 5% B in 5 C.V. and held at 5%B for 10 C.V. Total gradient run time equals 120 C.V. Flow rate 6.0 mL/min. Wavelength detection range: 254 nm. Column: Agilent AX1372-1 SF10-5.5gC8.

[0459] Preparative HPLC: Reverse-phase ultra-high-performance liquid chromatography (UPLC) was carried out on Phenomenex Gemini NX 5µ C-18 columns of the following dimensions: 150 x 4.6 mm for analysis, and 150 x 21.20 mm for preparative work. All UPLC experiments were performed with gradient conditions. Eluents used were solvent A (H_2O with 0.1% Formic acid) and solvent B (CH_3CN with 0.1% Formic acid). Flow rates used were 1.0 ml/min for analytical, and 20.0 ml/min for preparative HPLC. Detection was at 254 and 280 nm.

[0460] Synthesis of Intermediate 12



(a) 1',3'-Bis[2-methoxy-4-(methoxycarbonyl)phenoxy]propane ***(3)***

[0461] Diisopropyl azodicarboxylate (71.3 mL, 73.2 g, 362 mmol) was added drop-wise over a period of 60 min to an overhead stirred solution of methyl vanillate **2** (60.0 g, 329 mmol) and Ph_3P (129.4 g, 494 mmol) in anhydrous THF (800 mL) at 0-5°C (ice/acetone) under a

nitrogen atmosphere. The reaction mixture was allowed to stir at 0-5°C for an additional 1 hour after which time a solution of 1,3-propanediol (11.4 mL, 12.0 g, 158 mmol) in THF (12 mL) was added drop-wise over a period of 20 min. The reaction mixture was allowed to warm to room temperature and stirred for 5 days. The resulting white precipitate **3** was collected by vacuum filtration, washed with THF and dried in a vacuum desiccator to constant weight. Yield = 54.7 g (84% based on 1,3-propanediol). Purity satisfactory by LC/MS (3.20 min (ES+) *m/z* (relative intensity) 427 ($[M + Na]^{+\cdot}$, 10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (dd, 2H, J = 1.8, 8.3 Hz), 7.54 (d, 2H, J = 1.8 Hz), 6.93 (d, 2H, J = 8.5 Hz), 4.30 (t, 4H, J = 6.1 Hz), 3.90 (s, 6H), 3.89 (s, 6H), 2.40 (p, 2H, J = 6.0 Hz).

(b) 1',3'-Bis[2-methoxy-4-(methoxycarbonyl)-5-nitrophenoxy]propane ***(4)***

[0462] Solid $Cu(NO_3)_2.3H_2O$ (81.5 g, 337.5 mmol) was added slowly to an overhead stirred slurry of the bis-ester **3** (54.7 g, 135 mmol) in acetic anhydride (650 mL) at 0-5°C (ice/acetone). The reaction mixture was allowed to stir for 1 hour at 0-5°C and then allowed to warm to room temperature. A mild exotherm (*ca.* 40-50°C), accompanied by thickening of the mixture and evolution of NO_2 was observed at this stage. Additional acetic anhydride (300 mL) was added and the reaction mixture was allowed to stir for 16 hours at room temperature. The reaction mixture was poured on to ice (~ 1.5 L), stirred and allowed to return to room temperature. The resulting yellow precipitate was collected by vacuum filtration and dried in a desiccator to afford the desired *bis*-nitro compound **4** as a yellow solid. Yield = 66.7 g (100%). Purity satisfactory by LC/MS (3.25 min (ES+) *m/z* (relative intensity) 517 ($[M + Na]^{+\cdot}$, 40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (s, 2H), 7.06 (s, 2H), 4.32 (t, 4H, J = 6.0 Hz), 3.95 (s, 6H), 3.90 (s, 6H), 2.45–2.40 (m, 2H).

*(c) 1',3'-Bis(4-carboxy-2-methoxy-5-nitrophenoxy) propane (**5**)*

[0463] A slurry of the methyl ester **4** (66.7 g, 135 mmol) in THF (700 mL) was treated with 1N NaOH (700 mL) and the reaction mixture was allowed to stir vigorously at room temperature. After 4 days stirring, the slurry became a dark coloured solution which was subjected to rotary evaporation under reduced pressure to remove THF. The resulting aqueous residue was acidified to pH 1 with concentrated HCl and the colourless precipitate **5** was collected and dried thoroughly in a vacuum oven (50 °C). Yield = 54.5 g (87%). Purity satisfactory by LC/MS (2.65 min (ES+) *m/z* (relative intensity) 489 ($[M + Na]^{+\cdot}$, 30)); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.62 (s, 2H), 7.30 (s, 2H), 4.29 (t, 4H, J = 6.0 Hz), 3.85 (s, 6H), 2.30–2.26 (m, 2H).

*(d) 1,1'-[[(Propane-1,3-diyl)dioxy]bis[(5-methoxy-2-nitro-1,4-phenylene)carbonyl]]bis[(2S,4R)-methyl-4-hydroxypyrrolidine-2-carboxylate] **(6)***

[0464] Oxalyl chloride (24.5 mL, 35.6 g, 281 mmol) was added to a stirred suspension of the nitrobenzoic acid **5** (43 g, 92.3 mmol) and DMF (6 mL) in anhydrous DCM (600mL). Following initial effervescence the reaction suspension became a solution and the mixture was allowed to stir at room temperature for 16 hours. Conversion to the acid chloride was confirmed by treating a sample of the reaction mixture with MeOH and the resulting *bis*-methyl ester was observed by LC/MS. The majority of solvent was removed by evaporation under reduced pressure; the resulting concentrated solution was re-dissolved in a minimum amount of dry DCM and triturated with diethyl ether. The resulting yellow precipitate was collected by filtration, washed with cold diethyl ether and dried for 1 hour in a vacuum oven at 40°C. The solid acid chloride was added portionwise over a period of 25 min to a stirred suspension of (2*S*,4*R*)-

methyl-4-hydroxypyrrolidine-2-carboxylate hydrochloride (38.1 g, 210 mmol) and TEA (64.5 mL, g, 463 mmol) in DCM (400mL) at -40°C (dry ice/CH_3CN). Immediately, the reaction was complete as judged by LC/MS (2.47 min (ES+) *m/z* (relative intensity) 721 ($[M + H]^{+\cdot}$, 100). The mixture was diluted with DCM (200 mL) and washed with 1N HCl (300 mL), saturated $NaHCO_3$ (300 mL), brine (400 mL), dried ($MgSO_4$), filtered and the solvent evaporated *in vacuo* to give the pure product **6** as an orange solid (66.7 g, 100%). $[\alpha]^{22}_D$ = -46.1° (*c* = 0.47, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) (rotamers) δ 7.63 (s, 2H), 6.82 (s, 2H), 4.79–4.72 (m, 2H), 4.49–4.28 (m, 6H), 3.96 (s, 6H), 3.79 (s, 6H), 3.46–3.38 (m, 2H), 3.02 (d, 2H, *J* = 11.1 Hz), 2.48–2.30 (m, 4H), 2.29–2.04 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) (rotamers) δ 172.4, 166.7, 154.6, 148.4, 137.2, 127.0, 109.7, 108.2, 69.7, 65.1, 57.4, 57.0, 56.7, 52.4, 37.8, 29.0; IR (ATR, $CHCl_3$) 3410 (br), 3010, 2953, 1741, 1622, 1577, 1519, 1455, 1429, 1334, 1274, 1211, 1177, 1072, 1050, 1008, 871 cm^{-1}; MS (ES^+) *m/z* (relative intensity) 721 ($[M + H]^{+\cdot}$, 47), 388 (80); HRMS $[M + H]^{+\cdot}$ theoretical $C_{31}H_{36}N_4O_{16}$ *m/z* 721.2199, found (ES^+) *m/z* 721.2227.

(e) 1,1'-[[(Propane-1,3-diyl)dioxy]bis(11aS,2R)-2-(hydroxy)-7-methoxy-1,2,3,10,11,11a-hexahydro-5H-pyrrolo[2,1-c][1,4]-benzodiazepin-5,11-dione] (7)

[0465] **Method A:** A solution of the nitro-ester **6** (44 g, 61.1 mmol) in MeOH (2.8 L) was added to freshly purchased Raney® nickel (~ 50 g of a ~ 50% slurry in H_2O) and anti-bumping granules in a 5L 3-neck round bottomed flask. The mixture was heated at reflux and then treated dropwise with a solution of hydrazine hydrate (21.6 mL, 22.2 g, 693 mmol) in MeOH (200 mL) at which point vigorous effervescence was observed. When the addition was complete (~ 45 min) additional Raney® nickel was added carefully until effervescence had ceased and the initial yellow colour of the reaction mixture was discharged. The mixture was heated at reflux for a further 5 min at which point the reaction was deemed complete by TLC

(90:10 v/v $CHCl_3$/MeOH) and LC/MS (2.12 min (ES+) *m/z* (relative intensity) 597 ($[M + H]^{+\cdot}$, 100)). The reaction mixture was filtered hot immediately through a sinter funnel containing celite with vacuum suction. The filtrate was reduced in volume by evaporation *in vacuo* at which point a colourless precipitate formed which was collected by filtration and dried in a vacuum desiccator to provide **7** (31 g, 85%). $[\alpha]^{27}_D = +404°$ (*c* = 0.10, DMF); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.2 (s, 2H, N*H*), 7.26 (s, 2H), 6.73 (s, 2H), 5.11 (d, 2H, *J* = 3.98 Hz, O*H*), 4.32–4.27 (m, 2H), 4.19–4.07 (m, 6H), 3.78 (s, 6H), 3.62 (dd, 2H, *J* = 12.1, 3.60 Hz), 3.43 (dd, 2H, *J* = 12.0, 4.72 Hz), 2.67–2.57 (m, 2H), 2.26 (p, 2H, *J* = 5.90 Hz), 1.99–1.89 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.1, 164.0, 149.9, 144.5, 129.8, 117.1, 111.3, 104.5, 54.8, 54.4, 53.1, 33.5, 27.5; IR (ATR, neat) 3438, 1680, 1654, 1610, 1605, 1516, 1490, 1434, 1379, 1263, 1234, 1216, 1177, 1156, 1115, 1089, 1038, 1018, 952, 870 cm^{-1}; MS (ES^+) *m/z* (relative intensity) 619 ($[M + Na]^{+\cdot}$, 10), 597 ($[M + H]^{+\cdot}$, 52), 445 (12), 326 (11); HRMS $[M + H]^{+\cdot}$ theoretical $C_{29}H_{32}N_4O_{10}$ *m/z* 597.2191, found (ES^+) *m/z* 597.2205.

[0466] **Method B:** A suspension of 10% Pd/C (7.5 g, 10% w/w) in DMF (40 mL) was added to a solution of the nitro-ester **6** (75 g, 104 mmol) in DMF (360 mL). The suspension was hydrogenated in a Parr hydrogenation apparatus over 8 hours. Progress of the reaction was monitored by LC/MS after the hydrogen uptake had stopped. Solid Pd/C was removed by filtration and the filtrate was concentrated by rotary evaporation under vacuum (below 10mbar) at 40°C to afford a dark oil containing traces of DMF and residual charcoal. The residue was digested in EtOH (500 mL) at 40°C on a water bath (rotary evaporator bath) and the resulting suspension was filtered through celite and washed with ethanol (500 mL) to give a clear filtrate. Hydrazine hydrate (10 mL, 321 mmol) was added to the solution and the reaction mixture was heated at reflux. After 20 minutes the formation of a white precipitate was observed and reflux

was allowed to continue for a further 30 minutes. The mixture was allowed to cool down to room temperature and the precipitate was retrieved by filtration, washed with diethyl ether (2:1 volume of precipitate) and dried in a vacuum desiccator to provide **7** (50 g, 81%). Analytical data for method B: Identical to those obtained for Method A (optical rotation, ^{1}H NMR, LC/MS and TLC).

(f) 1,1'-[[(Propane-1,3-diyl)dioxy]bis(11aS,2R)-2-(tert-butyldimethylsilyloxy)-7-methoxy-1,2,3,10,11,11a-hexahydro-5H-pyrrolo[2,1-c][1,4]-benzodiazepin-5,11-dione]

[0467] TBSCl (27.6 g, 182.9 mmol) and imidazole (29.9 g, 438.8 mmol) were added to a cloudy solution of the tetralactam **7** (21.8 g, 36.6 mmol) in anhydrous DMF (400 mL) at 0°C (ice/acetone). The mixture was allowed to stir under a nitrogen atmosphere for 3 hours after which time the reaction was deemed complete as judged by LC/MS (3.90 min (ES+) *m/z* (relative intensity) 825 ([*M* + H]$^{+\cdot}$, 100). The reaction mixture was poured onto ice (~ 1.75 L) and allowed to warm to room temperature with stirring. The resulting white precipitate was collected by vacuum filtration, washed with H_2O, diethyl ether and dried in the vacuum desicator to provide pure **8** (30.1 g, 99%). $[\alpha]^{23}_D$ = +234° (*c* = 0.41, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (s, 2H, N*H*), 7.44 (s, 2H), 6.54 (s, 2H), 4.50 (p, 2H, *J* = 5.38 Hz), 4.21–4.10 (m, 6H), 3.87 (s, 6H), 3.73–3.63 (m, 4H), 2.85–2.79 (m, 2H), 2.36–2.29 (m, 2H), 2.07-1.99 (m, 2H), 0.86 (s, 18H), 0.08 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.4, 165.7, 151.4, 146.6, 129.7, 118.9, 112.8, 105.3, 69.2, 65.4, 56.3, 55.7, 54.2, 35.2, 28.7, 25.7, 18.0, -4.82 and -4.86; IR (ATR, $CHCl_3$) 3235, 2955, 2926, 2855, 1698, 1695, 1603, 1518, 1491, 1446, 1380, 1356, 1251, 1220, 1120, 1099, 1033 cm^{-1}; MS (ES$^+$) *m/z* (relative intensity) 825 ([*M* + H]$^{+\cdot}$, 62), 721 (14), 440 (38); HRMS [*M* + H]$^{+\cdot}$ theoretical $C_{41}H_{60}N_4O_{10}Si_2$ *m/z* 825.3921, found (ES$^+$) *m/z* 825.3948.

(g) 1,1'-[[(Propane-1,3-diyl)dioxy]bis(11aS,2R)-2-(tert-butyldimethylsilyloxy)-7-methoxy-10-((2-(trimethylsilyl)ethoxy)methyl)-1,2,3,10,11,11a-hexahydro-5H-pyrrolo[2,1-c][1,4]-benzodiazepin-5,11-dione] ***(9)***

[0468] A solution of *n*-BuLi (68.3 mL of a 1.6 M solution in hexane, 109 mmol) was added dropwise to a stirred suspension of the tetralactam **8** (30.08 g, 36.4 mmol) in anhydrous THF (600 mL) at -30°C (dry ice/ethylene glycol) under a nitrogen atmosphere. The reaction mixture was allowed to stir at this temperature for 1 hour (now a reddish orange colour) at which point a solution of SEMCl (19.3 mL, 18.2 g, 109 mmol) in anhydrous THF (120 mL) was added dropwise. The reaction mixture was allowed to slowly warm to room temperature and was stirred for 16 hours under a nitrogen atmosphere. The reaction was deemed complete as judged by TLC (EtOAc) and LC/MS (4.77 min (ES+) *m/z* (relative intensity) 1085 ($[M + H]^{+\cdot}$, 100). The THF was removed by evaporation *in vacuo* and the resulting residue dissolved in EtOAc (750 mL), washed with H_2O (250 mL), brine (250 mL), dried ($MgSO_4$) filtered and evaporated *in vacuo* to provide the crude N10-SEM-protected tetralactam **9** as an oil (max^m 39.5 g, 100%). Product carried through to next step without purification. $[\alpha]^{23}_D = +163°$ (*c* = 0.41, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (s, 2H), 7.22 (s, 2H), 5.47 (d, 2H, *J* = 9.98 Hz), 4.68 (d, 2H, *J* = 9.99 Hz), 4.57 (p, 2H, *J* = 5.77 Hz), 4.29–4.19 (m, 6H), 3.89 (s, 6H), 3.79–3.51 (m, 8H), 2.87–2.81 (m, 2H), 2.41 (p, 2H, *J* = 5.81 Hz), 2.03–1.90 (m, 2H), 1.02–0.81 (m, 22H), 0.09 (s, 12H), 0.01 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 165.7, 151.2, 147.5, 133.8, 121.8, 111.6, 106.9, 78.1, 69.6, 67.1, 65.5, 56.6, 56.3, 53.7, 35.6, 30.0, 25.8, 18.4, 18.1, -1.24, -4.73; IR (ATR, $CHCl_3$) 2951, 1685, 1640, 1606, 1517, 1462, 1433, 1360, 1247, 1127, 1065 cm^{-1}; MS (ES^+) *m/z* (relative intensity) 1113 ($[M + Na]^{+\cdot}$, 48), 1085 ($[M + H]^{+\cdot}$, 100), 1009 (5), 813 (6); HRMS $[M + H]^{+\cdot}$ theoretical $C_{53}H_{88}N_4O_{12}Si_4$ *m/z* 1085.5548, found (ES^+) *m/z* 1085.5542.

(h) 1,1'-[[(Propane-1,3-diyl)dioxy]bis(11aS,2R)-2-hydroxy-7-methoxy-10-((2-(trimethylsilyl)ethoxy)methyl)-1,2,3,10,11,11a-hexahydro-5H-pyrrolo[2,1-c][1,4]-benzodiazepin-5,11-dione] ***(10)***

[0469] A solution of TBAF (150 mL of a 1.0 M solution in THF, 150 mmol) was added to a stirred solution of the crude bis-silyl ether **9** [84.0 g (max^m 56.8 g), 52.4 mmol] in THF (800 mL) at room temperature. After stirring for 1 hour, analysis of the reaction mixture by TLC (95:5 v/v $CHCl_3$/MeOH) revealed completion of reaction. The THF was removed by evaporation under reduced pressure at room temperature and the resulting residue dissolved in EtOAc (500 mL) and washed with NH_4Cl (300 mL). The combined organic layers were washed with brine (60 mL), dried ($MgSO_4$), filtered and evaporated under reduced pressure to provide the crude product. Purification by flash chromatography (gradient elution: 100% $CHCl_3$ to 96:4 v/v $CHCl_3$/MeOH) gave the pure tetralactam **10** as a white foam (36.0 g, 79%). LC/MS 3.33 min (ES+) *m/z* (relative intensity) 879 ($[M + Na]^{+\cdot}$, 100), 857 ($[M + H]^{+\cdot}$, 40); $[\alpha]^{23}_D$ = +202° (*c* = 0.34, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.28 (s, 2H), 7.20 (s, 2H), 5.44 (d, 2H, *J* = 10.0 Hz), 4.72 (d, 2H, *J* = 10.0 Hz), 4.61–4.58 (m, 2H), 4.25 (t, 4H, *J* = 5.83 Hz), 4.20–4.16 (m, 2H), 3.91–3.85 (m, 8H), 3.77–3.54 (m, 6H), 3.01 (br s, 2H, O*H*), 2.96–2.90 (m, 2H), 2.38 (p, 2H, *J* = 5.77 Hz), 2.11–2.05 (m, 2H), 1.00–0.91 (m, 4H), 0.00 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 165.9, 151.3, 147.4, 133.7, 121.5, 111.6, 106.9, 79.4, 69.3, 67.2, 65.2, 56.5, 56.2, 54.1, 35.2, 29.1, 18.4, -1.23; IR (ATR, $CHCl_3$) 2956, 1684, 1625, 1604, 1518, 1464, 1434, 1361, 1238, 1058, 1021 cm^{-1}; MS (ES^+) *m/z* (relative intensity) 885 ($[M + 29]^{+\cdot}$, 70), 857 ($[M + H]^{+\cdot}$, 100), 711 (8), 448 (17); HRMS $[M + H]^{+\cdot}$ theoretical $C_{41}H_{60}N_4O_{12}Si_2$ *m/z* 857.3819, found (ES^+) *m/z* 857.3826.

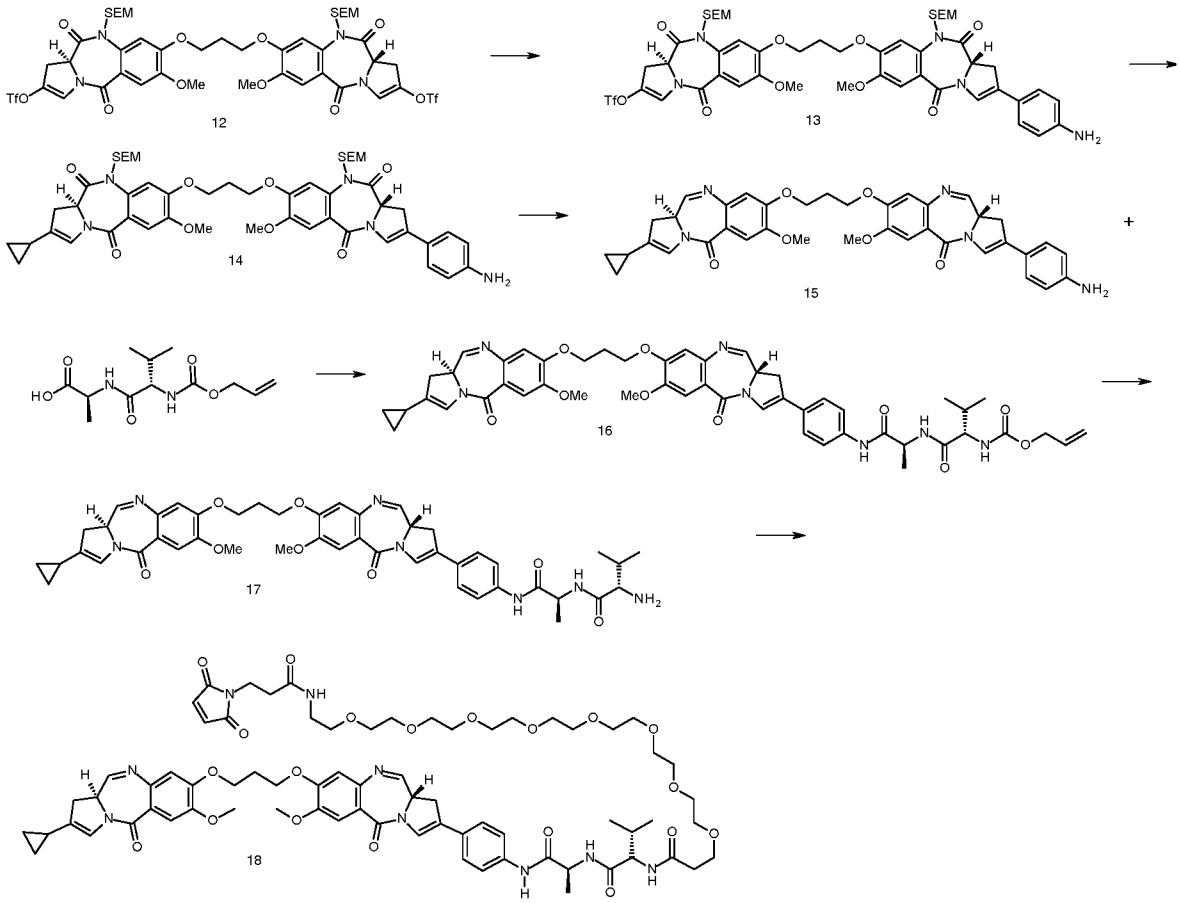
(i) 1,1'-[[(Propane-1,3-diyl)dioxy]bis(11aS)-7-methoxy-2-oxo-10-((2-(trimethylsilyl)ethoxy)methyl)-1,2,3,10,11,11a-hexahydro-5H-pyrrolo[2,1-c][1,4]-benzodiazepin-5,11-dione] ***(11)***

[0470] Diol **10** (25.6 g, 30 mmol, 1 eq.), NaOAc (6.9 g, 84 mmol, 2.8 eq.) and TEMPO (188 mg, 1.2 mmol, 0.04 eq.) were dissolved in DCM (326 mL) under Ar. This was cooled to -8°C (internal temperature) and TCCA (9.7 g, 42 mmol, 1.4 eq.) was added portionwise over 15 minutes. TLC (EtOAc) and LC/MS [3.60 min. (ES+) *m/z* (relative intensity) 854.21 ($[M + H]^{+\cdot}$, 40), (ES-) *m/z* (relative intensity) 887.07 ($[M - H + Cl]^{-\cdot}$, 10)] after 30 minutes indicated that reaction was complete. Cold DCM (200 mL) was added and the mixture was filtered through a pad of Celite before washing with a solution of saturated sodium hydrogen carbonate/ sodium thiosulfate (1:1 v/v; 200 mL x 2). The organic layer was dried with $MgSO_4$, filtered and the solvent removed *in vacuo* to yield a yellow/orange sponge (25.4 g, 99%). LC/MS [3.60 min. (ES+) *m/z* (relative intensity) 854.21 ($[M + H]^{+\cdot}$, 40); $[\alpha]^{20}_D = +291°$ (*c* = 0.26, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.32 (s, 2H), 7.25 (s, 2H), 5.50 (d, 2H, *J* = 10.1 Hz), 4.75 (d, 2H, *J* = 10.1 Hz), 4.60 (dd, 2H, *J* = 9.85, 3.07 Hz), 4.31–4.18 (m, 6H), 3.89–3.84 (m, 8H), 3.78–3.62 (m, 4H), 3.55 (dd, 2H, *J* = 19.2, 2.85 Hz), 2.76 (dd, 2H, *J* = 19.2, 9.90 Hz), 2.42 (p, 2H, *J* = 5.77 Hz), 0.98–0.91 (m, 4H), 0.00 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 206.8, 168.8, 165.9, 151.8, 148.0, 133.9, 120.9, 111.6, 107.2, 78.2, 67.3, 65.6, 56.3, 54.9, 52.4, 37.4, 29.0, 18.4, -1.24; IR (ATR, $CHCl_3$) 2957, 1763, 1685, 1644, 1606, 1516, 1457, 1434, 1360, 1247, 1209, 1098, 1066, 1023 cm^{-1}; MS (ES^+) *m/z* (relative intensity) 881 ($[M + 29]^{+\cdot}$, 38), 853 ($[M + H]^{+\cdot}$, 100), 707 (8), 542 (12); HRMS $[M + H]^{+\cdot}$ theoretical $C_{41}H_{56}N_4O_{12}Si_2$ *m/z* 853.3506, found (ES^+) *m/z* 853.3502.

(j) 1,1'-[[(Propane-1,3-diyl)dioxy]bis(11aS)-7-methoxy-2-[[(trifluoromethyl)sulfonyl]oxy]-10-((2-(trimethylsilyl)ethoxy)methyl)-1,10,11,11a-tetrahydro-5H-pyrrolo[2,1-c][1,4]-benzodiazepin-5,11-dione] ***(12)***

[0471] Anhydrous 2,6-lutidine (5.15 mL, 4.74 g, 44.2 mmol) was injected in one portion to a vigorously stirred solution of bis-ketone **11** (6.08 g, 7.1 mmol) in dry DCM (180 mL) at -45°C (dry ice/acetonitrile) under a nitrogen atmosphere. Anhydrous triflic anhydride, taken from a freshly opened ampoule (7.2 mL, 12.08 g, 42.8 mmol), was injected rapidly dropwise, while maintaining the temperature at -40°C or below. The reaction mixture was allowed to stir at -45°C for 1 hour at which point TLC (50/50 v/v *n*-hexane/EtOAc) revealed the complete consumption of starting material. The cold reaction mixture was immediately diluted with DCM (200 mL) and, with vigorous shaking, washed with water (1 x 100 mL), 5% citric acid solution (1 x 200 mL) saturated $NaHCO_3$ (200 mL), brine (100 mL) and dried ($MgSO_4$). Filtration and evaporation of the solvent under reduced pressure afforded the crude product which was purified by flash column chromatography (gradient elution: 90:10 v/v *n*-hexane/EtOAc to 70:30 v/v *n*-hexane/EtOAc) to afford bis-enol triflate **12** as a yellow foam (5.5 g, 70%). LC/MS 4.32 min (ES+) *m/z* (relative intensity) 1139 ($[M + Na]^{+\cdot}$, 20); $[\alpha]^{24}_D = +271°$ ($c = 0.18$, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (s, 2H), 7.26 (s, 2H), 7.14 (t, 2H, $J = 1.97$ Hz), 5.51 (d, 2H, $J = 10.1$ Hz), 4.76 (d, 2H, $J = 10.1$ Hz), 4.62 (dd, 2H, $J = 11.0$, 3.69 Hz), 4.32–4.23 (m, 4H), 3.94–3.90 (m, 8H), 3.81–3.64 (m, 4H), 3.16 (ddd, 2H, $J = 16.3$, 11.0, 2.36 Hz), 2.43 (p, 2H, $J = 5.85$ Hz), 1.23–0.92 (m, 4H), 0.02 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.1, 162.7, 151.9, 148.0, 138.4, 133.6, 120.2, 118.8, 111.9, 107.4, 78.6, 67.5, 65.6, 56.7, 56.3, 30.8, 29.0, 18.4, -1.25; IR (ATR, $CHCl_3$) 2958, 1690, 1646, 1605, 1517, 1456, 1428, 1360, 1327, 1207, 1136, 1096, 1060, 1022, 938, 913 cm^{-1}; MS (ES^+) *m/z* (relative intensity) 1144 ($[M + 28]^{+\cdot}$, 100), 1117 ($[M + H]^{+\cdot}$, 48), 1041 (40), 578 (8); HRMS $[M + H]^{+\cdot}$ theoretical $C_{43}H_{54}N_4O_{16}Si_2S_2F_6$ *m/z* 1117.2491, found (ES^+) *m/z* 1117.2465.

Example 11



*(a) (S)-8-(3-(((S)-2-(4-aminophenyl)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl trifluoromethanesulfonate (**13**)*

[0472] $Pd(PPh_3)_4$ (116.9 mg, 0.101 mmol) was added to a stirred mixture of the bis-enol triflate **12** (5.65 g, 5.06 mmol), 4-Aminophenylboronic acid pinacol ester (1 g, 4.56 mmol), Na_2CO_3 (2.46 g, 23.2 mmol), MeOH (37 mL), toluene (74 mL) and water (37 mL). The reaction mixture was allowed to stir at 30°C under a nitrogen atmosphere for 24 hours after which time all the boronic ester has consumed. The reaction mixture was then evaporated to dryness before

the residue was taken up in EtOAc (150 mL) and washed with H_2O (2 x 100 mL), brine (150 mL), dried ($MgSO_4$), filtered and evaporated under reduced pressure to provide the crude product. Purification by flash chromatography (gradient elution: 80:20 v/v Hexane/EtOAc to 60:40 v/v Hexane/EtOAc) afforded product **13** as a yellowish foam (2.4 g, 45%). LC/MS 4.02 min (ES+) *m/z* (relative intensity) 1060.21 ($[M + H]^{+\cdot}$, 100); ^{1}H–NMR: ($CDCl_3$, 400 MHz) δ 7.40 (s, 1H), 7.33 (s, 1H), 7.27 (bs, 3H), 7.24 (d, 2H, *J* = 8.5 Hz), 7.15 (t, 1H, *J* = 2.0 Hz), 6.66 (d, 2H, *J* = 8.5 Hz), 5.52 (d, 2H, *J* = 10.0 Hz), 4.77 (d, 1H, *J* = 10.0 Hz), 4.76 (d, 1H, *J* = 10.0 Hz), 4.62 (dd, 1H, *J* = 3.7, 11.0 Hz), 4.58 (dd, 1H, *J* = 3.4, 10.6 Hz), 4.29 (t, 4H, *J* = 5.6 Hz), 4.00-3.85 (m, 8H), 3.80 – 3.60 (m, 4H), 3.16 (ddd, 1H, *J* = 2.4, 11.0, 16.3 Hz), 3.11 (ddd, 1H, *J* = 2.2, 10.5, 16.1 Hz), 2.43 (p, 2H, *J* = 5.9 Hz), 1.1-0.9 (m, 4H), 0.2 (s, 18H). ^{13}C-NMR: ($CDCl_3$, 100 MHz) δ 169.8, 168.3, 164.0, 162.7, 153.3, 152.6, 149.28, 149.0, 147.6, 139.6, 134.8, 134.5, 127.9, 127.5, 125.1, 123.21, 121.5, 120.5, 120.1, 116.4, 113.2, 108.7, 79.8, 79.6, 68.7, 68.5, 67.0, 66.8, 58.8, 58.0, 57.6, 32.8, 32.0, 30.3, 19.7, 0.25.

*(b) (S)-2-(4-Aminophenyl)-8-(3-(((S)-2-cyclopropyl-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-10-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-5,11(10H,11aH)-dione (**14**)*

[0473] Triphenylarsine (0.24 g, 0.8 mmol), silver (I) oxide (1.02 g, 4.4 mmol), cyclopropylboronic acid (0.47 g, 5.5 mmol) and starting material **13** (1.15 g, 1.1 mmol) were dissolved in dioxane (30 mL) under an argon atmosphere. Potassium phosphate tribasic (2.8 g, 13.2 mmol) was ground-up with a pestle and mortar and quickly added to the reaction mixture. The reaction mixture was evacuated and flushed with argon 3 times and heated to 71°C. Palladium (II) *bis* (benzonitrile chloride) (84 mg, 0.22 mmol) was added and the reaction vessel was evacuated and flushed with argon 3 times. After 10 minutes a small sample was taken for

analysis by TLC (80:20 v/v ethyl acetate/hexane) and LC/MS. After 30 minutes the reaction had gone to completion (LC/MS analysis indicated complete consumption of starting material) and the reaction was filtered through celite and the filter pad washed with ethyl acetate (400 mL). The filtrate was washed with water (2 x 200 mL) and brine (2 x 200 mL). The organic layer was dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. Purification by silica gel column chromatography (30:70 v/v Hexane/ Ethyl acetate) afforded the product **14** as an orangey/yellow solid (0.66 g, 63%). Method 1, LC/MS (3.85 min (ES^+) *m/z* (relative intensity) 952.17 ($[M + H]^{+\cdot}$, 100). ^1H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, 2H, *J* = 8.4 Hz), 7.30 (s, 1H), 7.25 - 7.19 (m, 4H), 6.68 (s, 1H), 6.62 (d, 2H, *J* = 8.4 Hz), 5.49 (dd, 2H, *J* = 5.6, 10.0 Hz), 4.73 (app. t, 2H, *J* = 10.8 Hz), 4.54 (dd, 1H, *J* = 3.2, 10.4 Hz), 4.40 (dd, 1H, *J* = 3.2, 10.4 Hz), 4.29 - 4.23 (m, 4H), 3.91 - 3.85 (m, 7H), 3.80 - 3.71 (m, 2H), 3.70 - 3.61 (m, 2H), 3.38 - 3.32 (m, 1H), 3.12 - 3.01 (m, 1H), 2.50 - 2.69 (m, 1H), 2.40 (q, 2H, *J* = 5.6 Hz), 1.50 - 1.43 (m, 1H), 0.99 - 0.71 (m, 6H), 0.54 - 0.59 (m, 2H), 0.00 (s, 18H) ppm.

*(c) (S)-2-(4-Aminophenyl)-8-(3-(((S)-2-cyclopropyl-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-5(11aH)-one (**15**)*

[0474] SEM dilactam **14** (0.66 g, 0.69 mmol) was dissolved in THF (23 mL) and cooled to -78°C under an argon atmosphere. Super-Hydride® solution (1.7 mL, 1 M in THF) was added drop wise over 5 minutes while monitoring the temperature. After 20 minutes a small sample was taken and washed with water for LC/MS analysis. Water (50 mL) was added and the cold bath was removed. The organic layer was extracted and washed with brine (60 mL). The combined aqueous layers were washed with CH_2Cl_2/MeOH (90/10 v/v) (2 x 50 mL). The combined organic layers were dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. The crude product was dissolved in MeOH (48 mL), CH_2Cl_2 (18 mL) and water (6 mL) and sufficient silica gel was added to afford a thick suspension. After 5 days stirring, the suspension was filtered through a sintered funnel and washed with CH_2Cl_2/MeOH (9:1) (~ 200 mL) until product ceased to be eluted. The organic layer was washed with brine (2 x 70 mL), dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. Purification by silica gel column chromatography (100% $CHCl_3$ to 96/4 v/v $CHCl_3$/MeOH) afforded the product **15** as a yellow solid (302 mg, 66%). Method 1, LC/MS (2.42 min (ES^+) *m/z* (relative intensity) 660.74 ($[M + H]^{+\cdot}$, 30). ^1H NMR (400 MHz, $CDCl_3$) δ 7.86 (d, 1H, *J* = 3.6 Hz), 7.78 (d, 1H, *J* = 3.6 Hz), 7.58 – 7.44 (m, 3H), 7.34 – 7.20 (m, 3H), 6.88 – 6.66 (m, 4H), 4.35 – 4.15 (m, 6H), 3.95 – 3.75 (m, 7H), 3.39 – 3.22 (m, 1H), 3.14 – 3.04 (m, 1H), 2.93 - 2.85 (m, 1H), 2.46 – 2.36 (m, 2H), 1.49 – 1.41 (m, 1H), 0.80 – 0.72 (m, 2H), 0.58 – 0.51 (app. s, 2H) ppm.

*(d) Allyl ((2S)-1-(((2S)-1-((4-(8-(3-((2-cyclopropyl-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**16**)*

[0475] In a degassed round bottom flask filled with argon, HO-Ala-Val-alloc (149.6 mg, 0.549 mmol) and EEDQ (135.8 mg, 0.549 mmol) were dissolved in a 9:1 mixture of dry CH_2Cl_2/MeOH (5 mL). The flask was wrapped in aluminium foil and the reaction mixture was allowed to stir at room temperature for 1 hour before starting material **15** (302 mg, 0.457 mmol) was added. The reaction mixture was left to stir for a further 40 hours at room temperature before the volatiles were removed by rotary evaporation under reduced pressure (the reaction was followed by LC/MS, RT starting material 2.32 min, (ES^+ 660.29 ($[M+H]^{+\cdot}$,100)). The crude product was directly purified by silica gel chromatography column (100% $CHCl_3$ to 90/10 v/v $CHCl_3$/MeOH) to afford the pure product (**16**) in 42% yield (174 mg). Method 2 LC/MS (2.70 min (ES+) *m/z* (relative intensity) 914.73 ($[M+H]^{+\cdot}$, 60), 660.43 (60), 184.31 (100)).

*(e) (2S)-2-amino-N-((2S)-1-((4-(8-(3-((2-cyclopropyl-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**17**)*

[0476] The starting material **16** (170 mg, 0.185 mmol) was dissolved in dry CH_2Cl_2 (5 mL) in a round bottom flask filled with argon, before pyrrolidine (41 µL, 0.21 mmol) was added. The flask was purged/refilled three times with argon before $Pd(PPh_3)_4$ (14 mg, 0.084 mmol) was added and the flushing operation repeated. After 1 hour, complete consumption of starting material was observed (the reaction was followed by LC/MS) and Et_2O (50 mL) was added to the reaction mixture which was allowed to stir until all the product had crashed out of solution. The solid was filtered through a sintered funnel and washed twice with Et_2O (2 x 25 mL). The collecting flask was replaced and the isolated solid was dissolved in $CHCl_3$ (100 mL or until all the product had passed through the sintered funnel). The volatiles were then removed by rotary evaporation under reduced pressure to afford the crude product **17** which was used directly in the

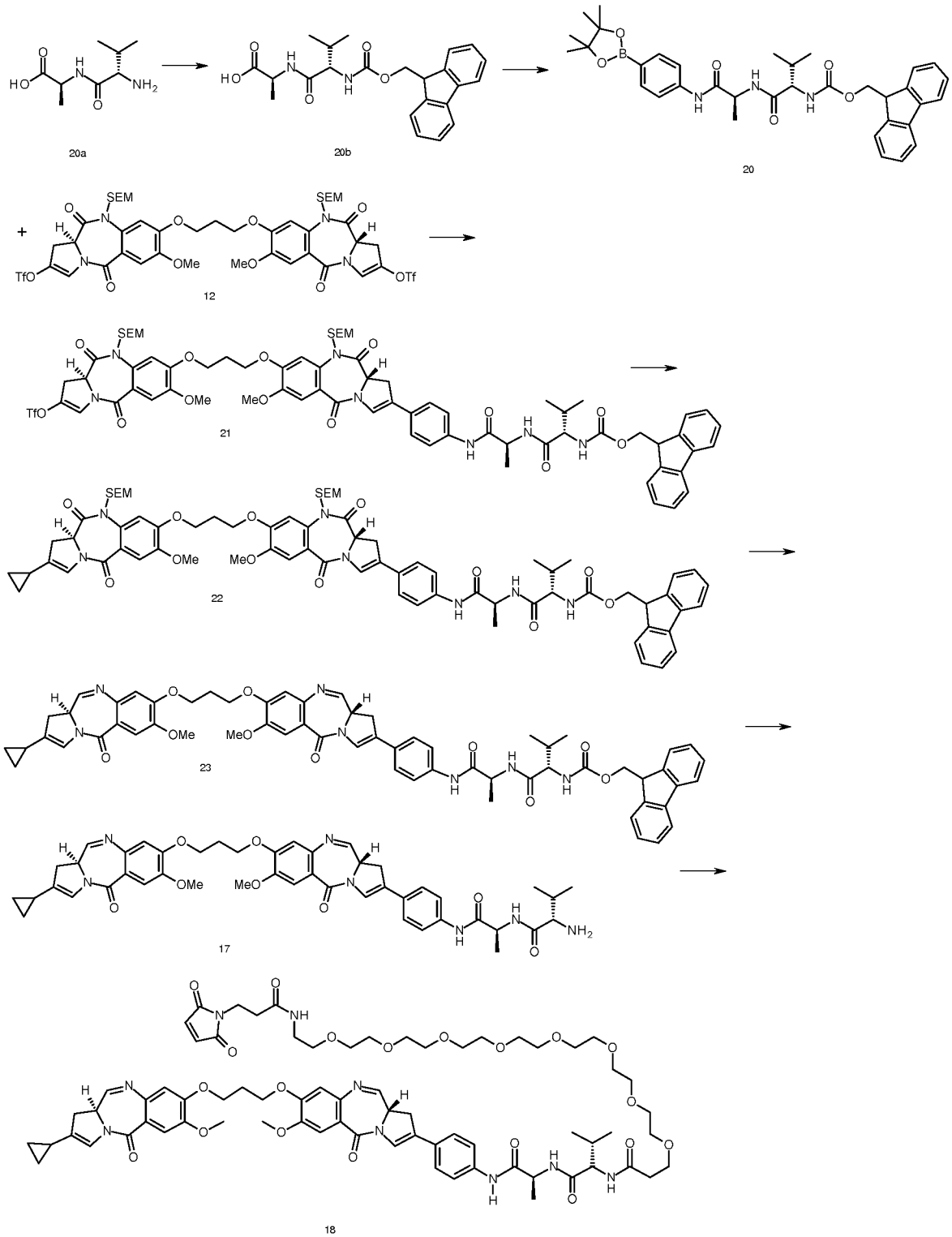
next step (168 mg). LC/MS method 2 (2.70 min (ES+) *m/z* (relative intensity) 830.27 ($[M+H]^{+\cdot}$, 50), 660.13 (80), 171.15 (100)).

*(f) N-((R)-1-(((S)-1-((4-((S)-8-(3-(((S)-2-cyclopropyl-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-1-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanamido)-3,6,9,12,15,18,21,24-octaoxaheptacosan-27-amide (**18**)*

[0477] Starting material **17** (154 mg, 0.185 mmol) and EDCI.HCl (110 mg, 0.185 mmol) were solubilised in dry CH_2Cl_2 (5 mL) in a round bottom flask purged and filled with argon. The mixture was left to stir at room temperature for 1 hour before PEG_8-maleimide (35.6 mg, 0.185 mmol) was added and the reaction mixture stirred for a further 16 hours (or until the reaction is complete, monitered by LC/MS). The reaction solution was diluted with CH_2Cl_2 (50 mL) and the organics were washed with H_2O (50 mL) and brine (50 mL) before being dried with $MgSO_4$, filtered and the solvent removed by rotary evaporation under reduced pressure to afford the crude product. Purification on silica gel column chromatography (100% $CHCl_3$ to 85/15 v/v $CHCl_3$/MeOH) gave the desired product (135mg), however remaining traces of unreacted PEG_8-maleimide were observed (by LC/MS, 2.21 min, method 2). Automated reverse phase silica gel chromatography (H_2O/CH_3CN) (see general information for conditions) successfully removed the impurity affording pure final product (**18**, 37mg of pure product starting from 110mg, 33%). Overall yield = 17%. Method 2 LC/MS (2.58 min (ES+) *m/z* (relative intensity) 1404.03 ($[M+H]^{+\cdot}$, 20), 702.63 (100)). ^1H NMR (400 MHz, $CDCl_3$) δ 7.91 (t, *J* = 3.5 Hz, 1H), 7.80 (d, *J* = 4.0 Hz, 1H), 7.75 (d, *J* = 8.8 Hz, 1H), 7.69 (d, *J* = 8.7 Hz, 1H), 7.54 – 7.50 (m, 2H), 7.45 (s, 1H), 7.39 – 7.31 (m, 2H), 6.87 (d, *J* = 10.5 Hz, 2H), 6.76 (s, 1H), 6.72 – 6.68 (m, 2H), 4.74 – 4.62 (m, 1H), 4.45 – 4.17 (m, 7H), 3.95 (s, 3H), 3.94 (s, 3H), 3.67 – 3.58 (m, 34H), 3.54 (m, 2H),

3.42 (dd, *J* = 10.2, 5.2 Hz, 2H), 3.16 – 3.07 (m, 1H), 2.92 (dd, *J* = 16.1, 4.1 Hz, 1H), 2.62 – 2.49 (m, 4H), 2.48 – 2.39 (m, 2H), 2.37 – 2.25 (m, 1H), 1.92 (s, 1H), 1.52 – 1.44 (m, 3H), 1.10 – 0.93 (m, 6H), 0.79 (dd, *J* = 9.2, 5.3 Hz, 2H), 0.57 (dd, *J* = 9.2, 5.3 Hz, 2H), NH were not observed.

Example 12



*(a) (R)-2-((R)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido) propanoic acid (**20b**)*

[0478] HO-Ala-Val-H **20a** (350 mg, 1.86 mmol) and Na_2CO_3 (493 mg, 4.65 mmol) were dissolved in distilled H_2O (15 mL) and the mixture was cooled to 0°C before dioxane (15 mL) was added (partial precipitation of the amino acid salt occurred). A solution of Fmoc-Cl (504 mg, 1.95 mmol) in dioxane (15 mL) was added dropwise with vigorous stirring over 10 minutes. The resulting mixture was stirred at 0°C for 2 hours before the ice bath was removed and stirring was maintained for 16 hours. The solvent was removed by rotary evaporation under reduced pressure and the residue dissolved in water (150 mL). The pH was adjusted from 9 to 2 with 1N HCl and the aqueous layer was subsequently extracted with EtOAc (3x100 mL). The combined organics were washed with brine (100 mL), dried with $MgSO_4$, filtered and the volatiles removed by rotary evaporation under reduced pressure to afford pure HO-Ala-Val-Fmoc **20b** (746 mg, 97% yield). LC/MS 2.85 min (ES+) *m/z* (relative intensity) 410.60 ; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J*=7.77 Hz, 2H), 7.60(d, *J*=7.77 Hz, 2H), 7.43(d, *J*=7.5 Hz, 2H), 7.34 (d, *J*=7.5 Hz, 2H), 6.30 (bs, 1H), 5.30 (bs, 1H), 4.71-7.56 (m, 1H), 4.54-4.36 (m, 2H), 4.08-3.91 (m, 1H), 2.21-2.07 (m, 1H), 1.50 (d, *J*=7.1 Hz, 3H), 1.06-0.90 (m, 6H).

*(b) (9H-fluoren-9-yl)methyl ((S)-3-methyl-1-oxo-1-(((S)-1-oxo-1-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)amino)propan-2-yl)amino)butan-2-yl)carbamate (**20**)*

[0479] 4-Aminophenylboronic acid pinacol ester was added (146.9 mg, 0.67 mmol) was added to a solution of HO-Ala-Val-Fmoc **20b** (330mg, 0.8 mmol), DCC (166 mg, 0.8 mmol) and DMAP (5 mg, cat.) in dry DCM (8 mL) previously stirred for 30 minutes at room temperature in a flask flushed with argon. The reaction mixture was then allowed to stir at room temperature overnight. The reaction was followed by LCMS and TLC. The reaction mixture was diluted with CH_2Cl_2 and the organics were washed with H_2O and brine before being dried with $MgSO_4$,

filtered and the solvent removed by rotary evaporation under reduced pressure. The crude product was dryloaded on a silicagel chromatography column (Hexane/EtOAc, 6:4) and pure product **20** was isolated as a white solid in 88% yield (360 mg).

*(c) 8-(3-((2-(4-((S)-2-((S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanamido)propanamido)phenyl)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl trifluoromethanesulfonate (**21**)*

[0480] Bis-triflate **12** (2.03g, 1.81 mmol), boronic pinacol ester (1g, 1.63 mmol) and Na_2CO_3 (881 mg, 8.31 mmol) were dissolved in a mixture of toluene/MeOH/H_2O, 2:1:1 (40 mL). The reaction flask was purged and filled with argon three times before *tetrakis*(triphenylphosphine)palladium(0) (41 mg, 0.035 mmol) was added and the reaction mixture heated to 30°C overnight. The solvents were removed under reduce pressure and the residue was taken up in H_2O (100 mL) and extracted with EtOAc (3 x 100 mL). The combined organics were washed with brine (100 mL), dried with $MgSO_4$, filtered and the volatiles removed by rotary evaporation under reduced pressure. The crude product was purified by silica gel chromatography column (Hexane/EtOAc, 8:2 to 25:75) to afford pure **21** in 33% yield (885 mg). LC/MS 3.85 min (ES+) *m/z* (relative intensity) 1452.90 ; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 – 7.16 (m, 17H), 7.13 (s, 1H), 6.51 – 6.24 (m, 1H), 5.51 (dd, *J* = 10.0, 5.1 Hz, 2H), 5.36 – 5.11 (m, 1H), 4.74 (dd, *J* = 10.1, 4.4 Hz, 2H), 4.70 – 4.53 (m, 2H), 4.47 (d, *J* = 6.4 Hz, 1H), 4.37 (d, *J* = 7.2 Hz, 1H), 4.27 (m, 4H), 4.20 – 4.14 (m, 1H), 3.90 (s, 3H), 3.89 (s, 3H), 3.77 (ddd, *J* = 16.7, 9.0, 6.4 Hz, 3H), 3.71 – 3.61 (m, 2H), 3.24 – 2.91 (m, 3H), 2.55 – 2.33 (m, 2H), 2.22 – 2.07 (m, 1H), 1.52 – 1.37 (m, 3H), 1.04 – 0.86 (m, 10H), 0.00 (s, 18H).

*(d) (9H-fluoren-9-yl)methyl((2S)-1-(((2S)-1-((4-(8-(3-((2-cyclopropyl-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**22**)*

[0481] Triphenylarsine (42 mg, 0.137 mmol) was added to a mixture of PBD-triflate **21** (250 mg, 0.172 mmol), cyclopropylboronic acid (73.9 mg, 0.86 mmol), silver oxide (159 mg, 0.688 mmol) and potassium phosphate tribasic (438 mg, 2.06 mmol) in dry dioxane (10 mL) under an argon atmosphere. The reaction was flushed with argon 3 times and *bis*(benzonitrile)palladium(II) chloride (13.2 mg, 0.034 mmol) was added. The reaction was flushed with Argon 3 more times before being warmed to 75°C and stirred for 10 minutes. The reaction mixture was filtered through a pad of celite which was subsequently rinsed with ethyl acetate. The solvent was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; 1 % methanol/chloroform). Pure fractions were collected and combined, and excess eluent was removed by rotary evaporation under reduced pressure to afford the desired product **22** (132 mg, 50 % yield). LC/MS 3.83 min (ES+) *m/z* (relative intensity) 1345.91 ; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 – 7.14 (m, 17H), 6.69 (s, 1H), 6.45 – 6.25 (m, 1H), 5.57 – 5.41 (m, 2H), 5.34 – 5.14 (m, 1H), 4.78 – 4.67 (m, 2H), 4.62 – 4.55 (m, 1H), 4.50 – 4.45 (m, 2H), 4.51 – 4.44 (m, 1H), 4.31 – 4.21 (m, 4H), 4.16 (m, 1H), 3.92 (s, 3H), 3.86 (s, 3H), 3.82 – 3.71 (m, 2H), 3.66 (m, 3H), 3.40 – 3.28 (m, 1H), 3.07 (m, 1H), 2.70 – 2.57 (m, 1H), 2.47 – 2.36 (m, 2H), 2.15 (m, 1H), 1.51 – 1.40 (m, 3H), 1.03 – 0.87 (m, 11H), 0.77 – 0.71 (m, 2H), 0.60 – 0.54 (m, 2H), 0.00 (t, J = 3.0 Hz, 18H).

(e) (9H-fluoren-9-yl)methyl((2S)-1-(((2S)-1-((4-(8-(3-((2-cyclopropyl-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5-

*oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**23**)*

[0482] A solution of Super-Hydride® (0.5 mL, 1M in THF) was added dropwise to a solution of SEM dilactam 22 (265 mg g, 0.19 mmol) in THF (10 mL) at -78°C under an argon atmosphere. The addition was completed over 5 minutes in order to maintain the internal temperature of the reaction mixture constant. After 20 minutes, an aliquot was quenched with water for LC/MS analysis, which revealed that the reaction was complete. Water (20 mL) was added to the reaction mixture and the cold bath was removed. The organic layer was extracted with EtOAc (3 x 30 mL) and the combined organics were washed with brine (50 mL), dried with $MgSO_4$, filtered and the solvent removed by rotary evaporation under reduced pressure. The crude product was dissolved in MeOH (12 mL), CH_2Cl_2 (6 mL), water (2 mL) and enough silica gel to form a thick stirring suspension. After 5 days, the suspension was filtered through a sintered funnel and washed with CH_2Cl_2/MeOH (9:1) (200 mL) until the elution of the product was complete. The organic layer was washed with brine (2 x 70 mL), dried with $MgSO_4$, filtered and the solvent removed by rotary evaporation under reduced pressure. Purification by silica gel column chromatography (100% $CHCl_3$ to 96% $CHCl_3$/ 4% MeOH) afforded the product 23 as a yellow solid (162 mg, 78%). LC/MS 3.02 min (ES+) *m/z* (relative intensity) 1052.37.

*(f) (2S)-2-amino-N-((2S)-1-((4-(8-(3-((2-cyclopropyl-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**17**)*

[0483] Excess piperidine was added (0.2 mL, 2 mmol) to a solution of SEM-dilactam **23** (76 mg, 0.073 mmol) in DMF (1 mL). The mixture was allowed to stir at room temperature for 20 min, at which point the reaction had gone to completion (as monitored by LC/MS). The

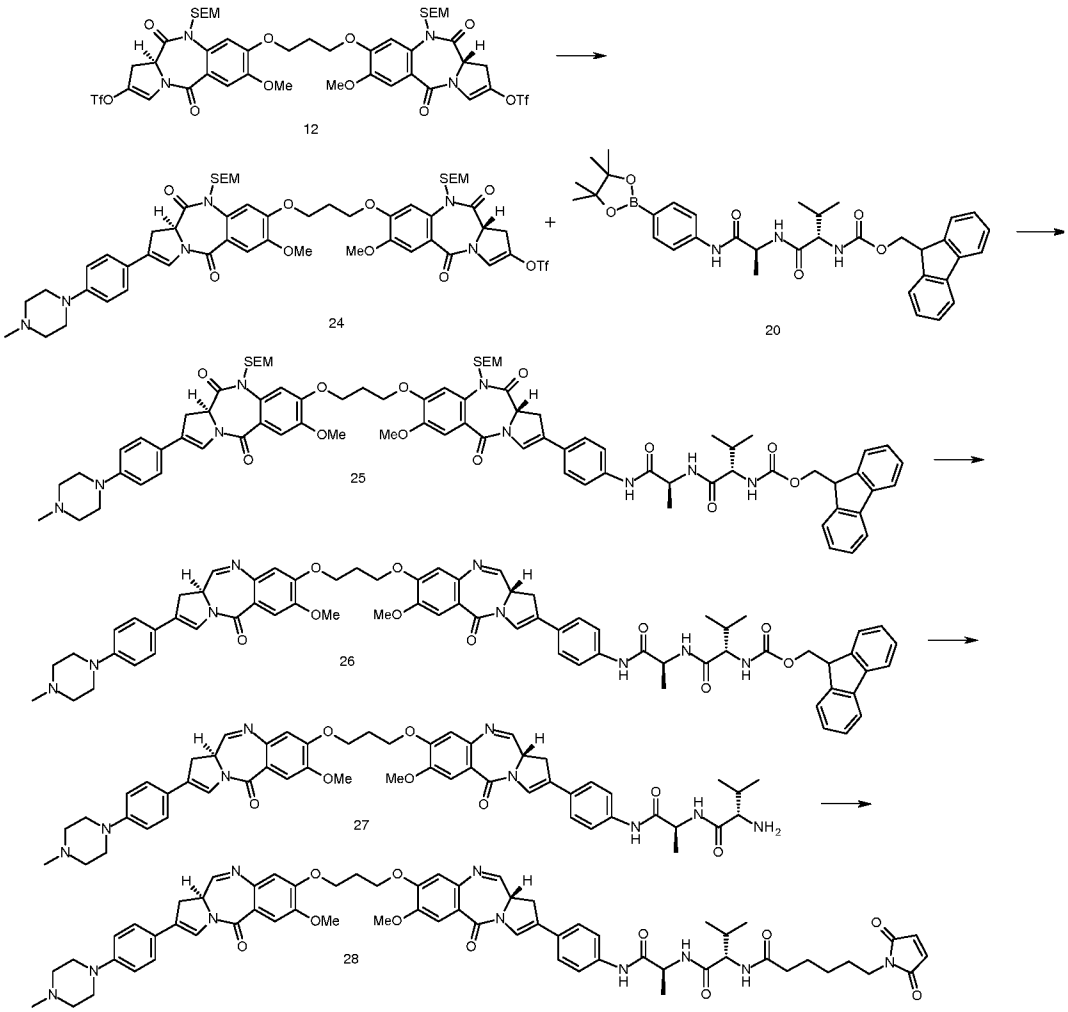
reaction mixture was diluted with CH_2Cl_2 (75 mL) and the organic phase was washed with H_2O (3x75 mL) until complete piperidine removal. The organic phase was dried over $MgSO_4$, filtered and excess solvent removed by rotary evaporation under reduced pressure to afford crude product **17** which was used as such in the next step. LC/MS 2.32 min (ES+) *m/z* (relative intensity) 830.00.

*(g) N-((2S)-1-(((2S)-1-((4-(8-(3-((2-cyclopropyl-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-1-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanamido)-3,6,9,12,15,18,21,24-octaoxaheptacosan-27-amide (**18**)*

[0484] EDCI hydrochloride (14 mg, 0.0732 mmol) was added to a suspension of Maleimide-PEG_8-acid (43.4 mg, 0.0732 mmol) in dry CH_2Cl_2 (5 mL) under argon atmosphere. The mixture was stirred for 1 hour at room temperature before PBD **17** (60.7 mg, 0.0732 mmol) was added. Stirring was maintained until the reaction was complete (usually 5 hours). The reaction was diluted with CH_2Cl_2 and the organic phase was washed with H_2O and brine before being dried over $MgSO_4$, filtered and excess solvent removed by rotary evaporation under reduced pressure by rotary evaporation under reduced pressure. The product was purified by careful silica gel chromatography (slow elution starting with 100% $CHCl_3$ up to 9:1 $CHCl_3$/MeOH) followed by reverse phase chromatography to remove unreacted maleimide-PEG_8-acid. The product **18** was isolated in 17.6% (21.8 mg). LC/MS 2.57 min (ES+) *m/z* (relative intensity) 1405.30 ; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (t, *J* = 3.5 Hz, 1H), 7.80 (d, *J* = 4.0 Hz, 1H), 7.75 (d, *J* = 8.8 Hz, 1H), 7.69 (d, *J* = 8.7 Hz, 1H), 7.54 – 7.50 (m, 2H), 7.45 (s, 1H), 7.39 – 7.31 (m, 2H), 6.87 (d, *J* = 10.5 Hz, 2H), 6.76 (s, 1H), 6.72 – 6.68 (m, 2H), 4.74 – 4.62 (m, 1H), 4.45 – 4.17 (m, 7H), 3.95 (s, 3H), 3.94 (s, 3H), 3.67 – 3.58 (m, 34H), 3.54 (m, 2H), 3.42

(dd, *J* = 10.2, 5.2 Hz, 2H), 3.16 – 3.07 (m, 1H), 2.92 (dd, *J* = 16.1, 4.1 Hz, 1H), 2.62 – 2.49 (m, 4H), 2.48 – 2.39 (m, 2H), 2.37 – 2.25 (m, 1H), 1.92 (s, 1H), 1.52 – 1.44 (m, 3H), 1.10 – 0.93 (m, 6H), 0.79 (dd, *J* = 9.2, 5.3 Hz, 2H), 0.57 (dd, *J* = 9.2, 5.3 Hz, 2H), NH were not observed.

Example 13



*(a) (**S**)-7-methoxy-8-(3-(((**S**)-7-methoxy-2-(4-(4-methylpiperazin-1-yl)phenyl)-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl trifluoromethanesulfonate (**24**)*

[0485] $Pd(PPh_3)_4$ (20.6 mg, 0.018 mmol) was added to a stirred mixture of the bis-enol triflate **12** (500 mg, 0.44 mmol), N-methyl piperazine boronic ester (100 mg, 0.4 mmol), Na_2CO_3 (218 mg, 2.05 mmol), MeOH (2.5 mL), toluene (5 mL) and water (2.5 mL). The reaction mixture

was allowed to stir at 30°C under a nitrogen atmosphere for 24 hours after which time all the boronic ester has consumed. The reaction mixture was then evaporated to dryness before the residue was taken up in EtOAc (100 mL) and washed with H_2O (2 x 50 mL), brine (50 mL), dried ($MgSO_4$), filtered and evaporated under reduced pressure to provide the crude product. Purification by flash chromatography (gradient elution: 80:20 v/v Hexane/EtOAc to 60:40 v/v Hexane/EtOAc) afforded product **24** as a yellowish foam (122.6 mg, 25%).

[0486] LC/MS 3.15 min (ES+) *m/z* (relative intensity) 1144 ($[M + H]^{+\cdot}$, 20%).

*(b) (9H-fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-((S)-7-methoxy-8-(3-(((S)-7-methoxy-2-(4-(4-methylpiperazin-1-yl)phenyl)-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**25**)*

[0487] PBD-triflate **24** (359 mg, 0.314 mmol), boronic pinacol ester **20** (250 mg, 0.408 mmol) and triethylamine (0.35 mL, 2.51 mmol) were dissolved in a mixture of toluene/MeOH/H_2O, 2:1:1 (3 mL). The microwave vessel was purged and filled with argon three times before *tetrakis*(triphenylphosphine)palladium(0) (21.7 mg, 0.018 mmol) was added and the reaction mixture placed in the microwave at 80°C for 10 minutes. Subsequently, CH_2Cl_2 (100 mL) was added and the organics were washed with water (2 x 50 mL) and brine (50 mL) before being dried with $MgSO_4$, filtered and the volatiles removed by rotary evaporation under reduced pressure. The crude product was purified by silica gel chromatography column ($CHCl_3$/MeOH, 100% to 9:1) to afford pure **25** (200 mg, 43% yield). LC/MS 3.27 min (ES+) *m/z* (relative intensity) 1478 ($[M + H]^{+\cdot}$, 100%).

*(c) (9H-fluoren-9-yl)methyl ((**S**)-1-(((**S**)-1-((4-((**S**)-7-methoxy-8-(3-(((**S**)-7-methoxy-2-(4-(4-methylpiperazin-1-yl)phenyl)-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-*

*c][1,4]benzodiazepin-8-yl)oxy)propoxy)-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**26**)*

[0488] A solution of Super-Hydride® (0.34 mL, 1M in THF) was added dropwise to a solution of SEM-dilactam **25** (200 mg, 0.135 mmol) in THF (5 mL) at -78°C under an argon atmosphere. The addition was completed over 5 minutes in order to maintain the internal temperature of the reaction mixture constant. After 20 minutes, an aliquot was quenched with water for LC/MS analysis, which revealed that the reaction was complete. Water (20 mL) was added to the reaction mixture and the cold bath was removed. The organic layer was extracted with EtOAc (3 x 30 mL) and the combined organics were washed with brine (50 mL), dried with $MgSO_4$, filtered and the solvent removed by rotary evaporation under reduced pressure. The crude product was dissolved in MeOH (6 mL), CH_2Cl_2 (3 mL), water (1 mL) and enough silica gel to form a thick stirring suspension. After 5 days, the suspension was filtered through a sintered funnel and washed with CH_2Cl_2/MeOH (9:1) (100 mL) until the elution of the product was complete. The organic layer was washed with brine (2 x 50 mL), dried with $MgSO_4$, filtered and the solvent removed by rotary evaporation under reduced pressure. Purification by silica gel column chromatography (100% $CHCl_3$ to 96% $CHCl_3$/ 4% MeOH) afforded the product **26** as a yellow solid (100 mg, 63%). LC/MS 2.67 min (ES+) *m/z* (relative intensity) 1186 ($[M + H]^{+\cdot}$, 5%).

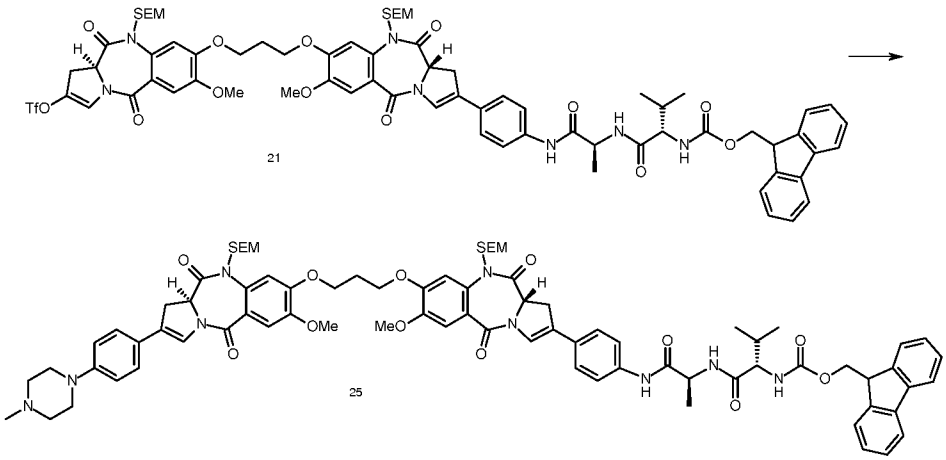
*(d) (**S**)-2-amino-N-((**S**)-1-((4-((**R**)-7-methoxy-8-(3-(((**R**)-7-methoxy-2-(4-(4-methylpiperazin-1-yl)phenyl)-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**27**)*

[0489] Excess piperidine was added (0.1 mL, 1 mmol) to a solution of PBD **26** (36.4 mg, 0.03 mmol) in DMF (0.9 mL). The mixture was allowed to stir at room temperature for 20 min, at which point the reaction had gone to completion (as monitored by LC/MS). The reaction mixture was diluted with CH_2Cl_2 (50 mL) and the organic phase was washed with H_2O (3 x 50 mL) until complete piperidine removal. The organic phase was dried over $MgSO_4$, filtered and excess solvent removed by rotary evaporation under reduced pressure to afford crude product **27** which was used as such in the next step. LC/MS 2.20 min (ES+) *m/z* (relative intensity) 964 ([*M* + H]$^{+\cdot}$, 5%).

*(e) 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-((**S**)-1-(((**S**)-1-((4-((**S**)-7-methoxy-8-(3-(((S)-7-methoxy-2-(4-(4-methylpiperazin-1-yl)phenyl)-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)hexanamide (**28**)*

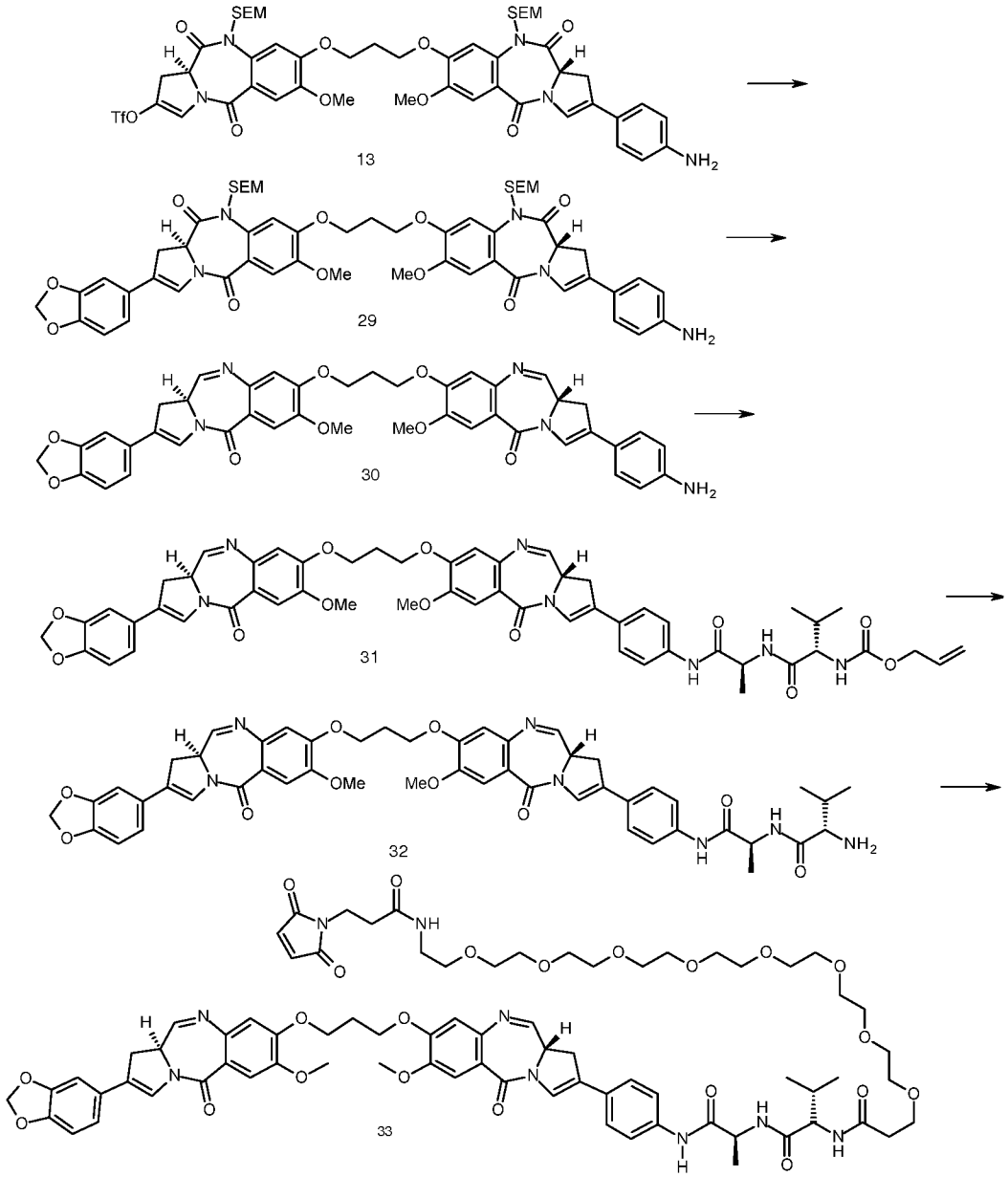
[0490] EDCI hydrochloride (4.7 mg, 0.03 mmol) was added to a suspension of 6-maleimidohexanoic acid (6.5 mg, 0.03 mmol) in dry CH2Cl2 (3 mL) under argon atmosphere. The mixture was stirred for 1 hour at room temperature before PBD 27 (34 mg, crude) was added. Stirring was maintained until the reaction was complete (6 hours). The reaction was diluted with CH2Cl2 and the organic phase was washed with H2O and brine before being dried over MgSO4, filtered and excess solvent removed by rotary evaporation under reduced pressure by rotary evaporation under reduced pressure. The product was purified by careful silica gel chromatography (slow elution starting with 100% CHCl3 up to 9:1 CHCl3/MeOH) followed by reverse phase chromatography to remove unreacted maleimide-PEG8-acid. The product 28 was isolated in 41% over two steps (14.6 mg). LC/MS 2.40 min (ES+) m/z (relative intensity) 1157 ([M + H]+., 5%).

Example 14



[0491] PBD-triflate **21** (469 mg, 0.323 mmol), boronic pinacol ester (146.5 mg, 0.484 mmol) and Na_2CO_3 (157 mg, 1.48 mmol) were dissolved in a mixture of toluene/MeOH/H_2O, 2:1:1 (10 mL). The reaction flask was purged with argon three times before *tetrakis*(triphenylphosphine)palladium(0) (7.41 mg, 0.0064 mmol) was added and the reaction mixture heated to 30°C overnight. The solvents were removed under reduced pressure and the residue was taken up in H_2O (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organics were washed with brine (100 mL), dried with $MgSO_4$, filtered and the volatiles removed by rotary evaporation under reduced pressure. The crude product was purified by silica gel column chromatography ($CHCl_3$ 100% to $CHCl_3$/MeOH 95%:5%) to afford pure **25** in 33% yield (885 mg). LC/MS 3.27 min (ES+) *m/z* (relative intensity) 1478 ($[M + H]^{+\cdot}$, 100%).

Example 15



*(a) (S)-2-(4-Aminophenyl)-8-(3-(((S)-2-(benzo[d][1,3]dioxol-5-yl)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-10-((2-(trimethylsilyl)ethoxy)methyl)-1H-pyrrolo[2,1-c][1,4]benzodiazepine-5,11(10H,11aH)-dione (**29**)*

[0492] 3, 4-(Methylenedioxy)phenyl boronic acid (356 mg, 2.1 mmol, 1.3 equiv.), TEA (1.8 mL, 12.9 mmol, 8 equiv.) and triflate/aniline **13** (1.75 g, 1.7 mmol, 1 equiv.) were dissolved in a mixture of ethanol (7 mL), toluene (13 mL) and water (2 mL) under an Ar atmosphere. The reaction mixture was evacuated and flushed with Ar 3 times, before addition of tetrakis(triphenylphosphine)palladium(0) (114 mg, 0.1 mmol, 0.06 equiv.). The flask was again evacuated and flushed with Ar 3 times and heated in a microwave at 80°C for 8 minutes with 30 seconds pre-stirring time. Analysis by TLC (80:20 v/v ethyl acetate/hexane) indicated complete consumption of starting material. The reaction mixture was diluted with dichloromethane (50 mL) and washed with water (50 mL). The organic layer was dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. Purification by silica gel column chromatography (60:40 to 20:80 v/v hexane/ ethyl acetate) afforded the product **29** as a yellow solid (1.21 g, 71%). LC/MS (3.92 min (ES^+) *m/z* (relative intensity) 1032.44 ($[M + H]^{+\cdot}$, 100).

*(b) (S)-2-(4-Aminophenyl)-8-(3-(((S)-2-(benzo[d][1,3]dioxol-5-yl)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-1H-pyrrolo[2,1-c][1,4]benzodiazepin-5(11aH)-one (**30**)*

[0493] SEM dilactam **29** (0.25 g, 0.24 mmol, 1 equiv.) was dissolved in THF (8 mL) and cooled to -78°C under an Ar atmosphere. Super-Hydride® (0.6 mL, 1 M in THF, 2.5 equiv.) was added drop wise over 5 minutes while monitoring the temperature. After 20 minutes a small sample was taken and worked-up for LCMS analysis. Water (50 mL) was added, the cold bath was removed and the solution washed with ethyl acetate (50 mL). The organic layer was extracted and washed with brine (60 mL), dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. The crude product was dissolved in EtOH (15 mL), CH_2Cl_2 (7.5 mL) and water (2.5 mL) and enough silica gel was added until it was a thick suspension. After 5 days stirring, it was filtered through a sintered funnel and washed with CH_2Cl_2/MeOH (9:1) (100 mL) until product

ceased to be eluted. The organic layer was washed with brine (2 x 50 mL), dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. Purification by silica gel column chromatography ($CHCl_3$ with 1% to 4% MeOH gradient) afforded the product **30** as a yellow solid (94 mg, 53%). LC/MS (2.53 min (ES^+) *m/z* (relative intensity) 739.64 ($[M]^{+\cdot}$, 70).

*(c) Allyl ((S)-1-(((S)-1-((4-((S)-8-(3-(((S)-2-(benzo[d][1,3]dioxol-5-yl)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**31**)*

[0494] Under an Ar atmosphere, Alanine-Valine-Alloc (180 mg, 0.66 mmol, 1.2 equiv.) was stirred with EEDQ (163 mg, 0.66 mmol, 1.2 equiv.) in anhydrous CH_2Cl_2 (21 mL) and methanol (1 mL) for 1 hour. The PBD **30** (407 mg, 0.55 mmol, 1 equiv.) was dissolved in anhydrous CH_2Cl_2 (21 mL) and methanol (1 mL) and added to the reaction. LC/MS after 5 days stirring at room temperature showed majority product formation. The solvent was removed *in vacuo* before purification by column chromatography (CH_2Cl_2 with 1% to 6% MeOH gradient) to yield the product **31** as a yellow solid (184 mg, 34%). LC/MS (2.95 min (ES^+) *m/z* (relative intensity) 994.95 ($[M + H]^{+\cdot}$, 60).

*(d) (S)-2-Amino-N-((S)-1-((4-((S)-8-(3-(((S)-2-(benzo[d][1,3]dioxol-5-yl)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**32**)*

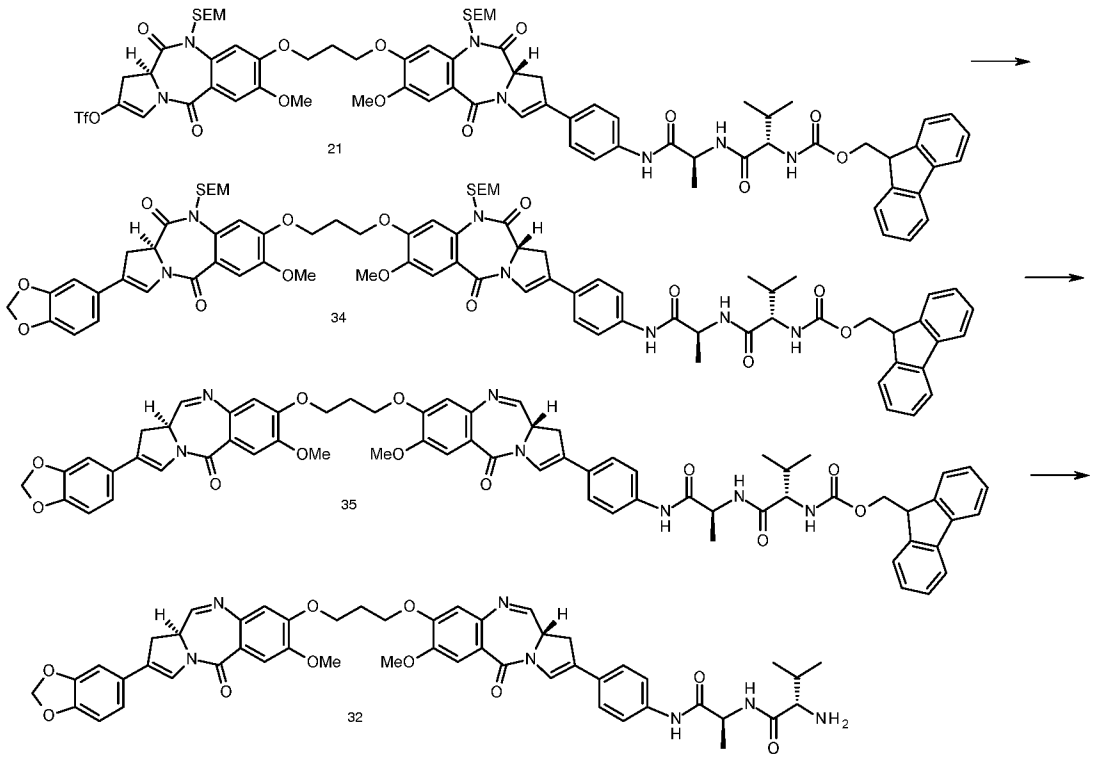
[0495] The imine **31** (100 mg, 0.1 mmol, 1 equiv.) was dissolved in anhydrous DCM (10 mL) (with the aid of one drop of methanol to aid dissolution) under an Ar atmosphere. Pyrrolidine (30 µL, 0.15 mmol, 1.5 equiv.) was added drop wise before the flask was evacuated and flushed with Ar three times. $Pd(PPh_3)_4$ (7 mg, 6 µmol, 0.06 equiv.) was added and the flask was evacuated and flushed with Ar three times. LC/MS analysis after 1 hour indicated product

formation and complete loss of starting material. Et_2O (60 mL) was added to the reaction mixture and it was left to stir until all the product had crashed out of solution. The precipitate was filtered through a sintered funnel and washed twice with Et_2O (2 x 20 mL). The collection flask was replaced and the isolated solid was dissolved and washed through the sinter with $CHCl_3$ (100 mL). The solvent was removed *in vacuo* to afford the crude product **32** as a yellow solid which was used directly in the next step. LC/MS (1.14 min (ES^+) *m/z* (relative intensity) 910.40 ([*M* + H]$^{+\cdot}$, 67).

*(e) N-((S)-1-(((S)-1-((4-((S)-8-(3-(((S)-2-(Benzo[d][1,3]dioxol-5-yl)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)-1-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanamido)-3,6,9,12,15,18,21,24-octaoxaheptacosan-27-amide (**33**)*

[0496] The imine **32** (92 mg, 0.1 mmol, 1.1 equiv.) was dissolved in $CHCl_3$ (6 mL) with one drop of anhydrous MeOH to aid dissolution. Maleimide-PEG_8-acid (53 mg, 0.09 mmol, 1 equiv.) was added followed by EEDQ (33 mg, 0.14 mmol, 1.5 equiv.). This was left to stir vigorously at room temperature under Ar for 4 days until LC/MS analysis showed majority product formation. The solvent was removed in vacuo and the crude product was partially purified by silica gel column chromatography (CHCl3 with 1% to 10% MeOH gradient) yielding **33** (81mg). The material was purified further by preparative HPLC to give **33** as a yellow solid (26.3 mg, 18%). Fast Formic run: LC/MS (1.39 min (ES+) m/z (relative intensity) 1485.00 ([M + H]+., 64).

Example 16



*(a) 9H-Fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-((S)-8-(3-(((S)-2-(benzo[d][1,3]dioxol-5-yl)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-5,11-dioxo-10-((2-(trimethylsilyl)ethoxy)methyl)-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**34**)*

[0497] The triflate **21** (0.5 g, 0.35 mmol, 1 equiv.), 3, 4-(methylenedioxy)phenyl boronic acid (75 mg, 0.45 mmol, 1.3 equiv.) and Na_2CO_3 (0.17 g, 1.6 mmol, 4.5 equiv.) were dissolved in toluene (11 mL), EtOH (5.5 mL) and water (5.5 mL) under an Ar atmosphere. The flask was evacuated and flushed with Ar three times. $Pd(PPh_3)_4$ (24 mg, 0.02 mmol, 0.06 equiv.) was added and again the flask was evacuated and flushed with Ar three times. This was heated to 30°C and left stirring overnight. Analysis by LC/MS showed complete loss of starting material. The

solvent was removed *in vacuo* and the residue dissolved in water (60 mL) before washing with ethyl acetate (60 mL x 3). The combined organic layers were washed with brine (50 mL), dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. Purification by column chromatography (50:50 to 25:75 v/v hexane/ ethyl acetate) afforded the product **34** as a yellow solid (310 mg, 64%). LC/MS (1.44 min (ES^-) *m/z* (relative intensity) 1423.35 ($[M - H]^-$, 79).

*(b) (9H-Fluoren-9-yl)methyl ((S)-1-(((S)-1-((4-((S)-8-(3-(((S)-2-(benzo[d][1,3]dioxol-5-yl)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**35**)*

[0498] SEM dilactam **34** (0.31 g, 0.22 mmol, 1 equiv.) was dissolved in THF (10 mL) and cooled to -78°C under an Ar atmosphere. Super-Hydride® (0.5 mL, 1 M in THF, 2.5 equiv.) was added drop wise over 5 minutes while monitoring the temperature. After 30 minutes a small sample was taken and worked-up for LC/MS analysis. Water (50 mL) was added, the cold bath was removed and the solution washed with ethyl acetate (50 mL). The organic layer was extracted and washed with brine (60 mL), dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. The crude product was dissolved in EtOH (13.2 mL), CH_2Cl_2 (6.6 mL) and water (2.2 mL) and enough silica gel was added until it was a thick suspension. After 5 days stirring, it was filtered through a sintered funnel and washed with CH_2Cl_2/MeOH (9:1) (100 mL) until product ceased to be eluted. The organic layer was washed with brine (2 x 50 mL), dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. Purification by silica gel column chromatography ($CHCl_3$ with 1% to 4% MeOH gradient) afforded the pure product **35** as a yellow solid (185 mg, 75%). LC/MS (1.70 min (ES^+) *m/z* (relative intensity) 1132.85 ($[M + H]^+$, 60).

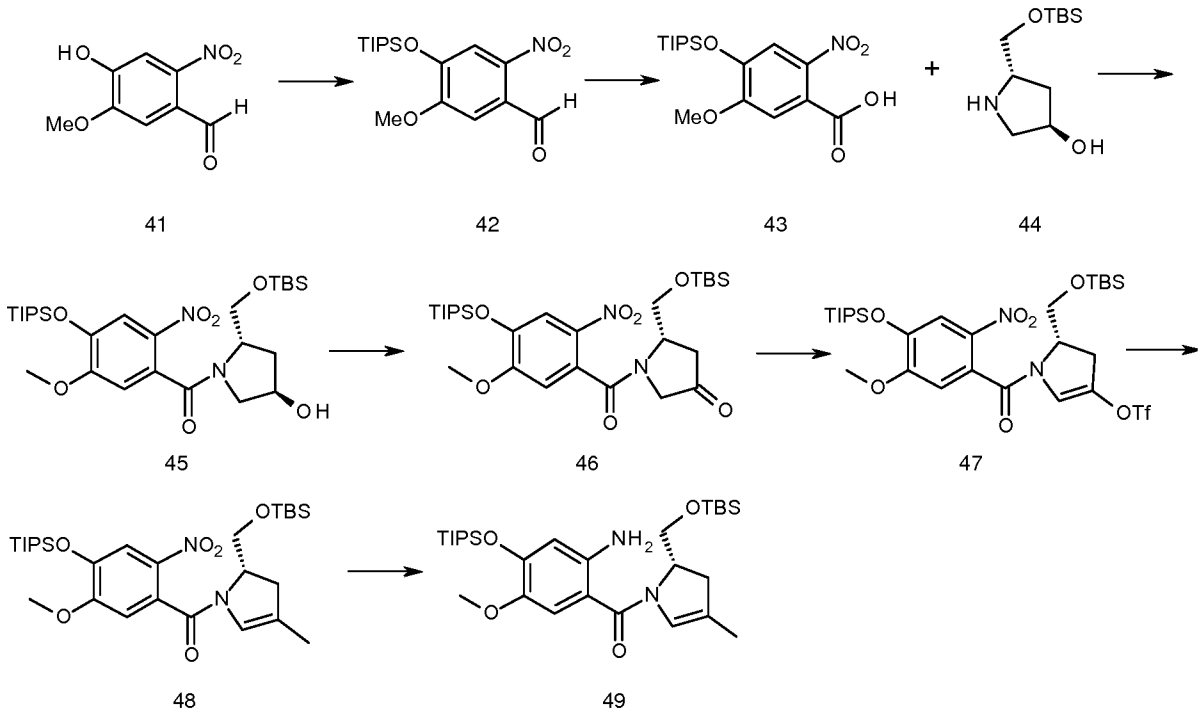
(c) (S)-2-Amino-N-((S)-1-((4-((S)-8-(3-(((S)-2-(benzo[d][1,3]dioxol-5-yl)-7-methoxy-5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)propoxy)-7-methoxy-

*5-oxo-5,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-2-yl)phenyl)amino)-1-oxopropan-2-yl)-3-methylbutanamide (**32**)*

[0499] The imine **35** (82 mg, 0.07 mmol, 1 equiv.) was dissolved in DMF (1 mL) before piperidine (0.2 mL, 2 mmol, excess) was added slowly. This solution was left to stir at room temperature for 20 minutes until LC/MS analysis showed complete consumption of starting material. The reaction mixture was diluted with CH_2Cl_2 (50 mL), washed with water (50 mL x 4), dried with $MgSO_4$, filtered and the solvent removed *in vacuo*. The product **33** was used without further purification in the next step. LC/MS (1.15 min (ES^+) *m/z* (relative intensity) 910.60 ($[M + H]^{+\cdot}$, 58).

Example 17

(i) (S)-(2-amino-5-methoxy-4-((triisopropylsilyl)oxy)phenyl)(2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone (49)



*(a) 5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)benzaldehyde (**42**)*

[0500] Neat triisopropylsilylchloride (56.4 mL, 262 mmol) was added to a mixture of imidazole (48.7 g, 715.23 mmol) and 4-hydroxy-5-methoxy-2-nitrobenzaldehyde **41** (47 g, 238 mmol) (ground together). The mixture was heated until the phenol and imidazole melted and went into solution (100 °C). The reaction mixture was allowed to stir for 15 minutes and was then allowed to cool, whereupon a solid was observed to form at the bottom of the flask (imidazole chloride). The reaction mixture was diluted with 5% EtOAc/ hexanes and loaded directly onto silica gel and the pad was eluted with 5% EtOAc/ hexanes , followed by 10% EtOAc/hexanes (due to the low excess, very little unreacted TIPSCl was found in the product). The desired product was eluted with 5 % ethyl acetate in hexane. Excess eluent was removed by

rotary evaporation under reduced pressure, followed by drying under high vacuum to afford a crystalline light sensitive solid (74.4 g, 88 %). Purity satisfactory by LC/MS (4.22 min (ES+) *m/z* (relative intensity) 353.88 ($[M + H]^{+\cdot}$, 100)); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.43 (s, 1H), 7.60 (s, 1H), 7.40 (s, 1H), 3.96 (s, 3H), 1.35 – 1.24 (m, 3H), 1.10 (m, 18H).

*(b) 5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)benzoic acid (**43**)*

[0501] A solution of sodium chlorite (47.3 g, 523 mmol, 80 % technical grade) and sodium dihydrogenphosphate monobasic (35.2 g, 293 mmol) (NaH_2PO_4) in water (800 mL) was added to a solution of compound **2** (74 g, 209 mmol) in tetrahydrofuran (500 mL) at room temperature. Hydrogen peroxide (60 % w/w, 140 mL, 2.93 mol) was immediately added to the vigorously stirred biphasic mixture. The reaction mixture evolved gas (oxygen), the starting material dissolved and the temperature of the reaction mixture rose to 45°C. After 30 minutes LC/MS revealed that the reaction was complete. The reaction mixture was cooled in an ice bath and hydrochloric acid (1 M) was added to lower the pH to 3 (this step was found unnecessary in many instances, as the pH at the end of the reaction is already acidic; please check the pH before extraction). The reaction mixture was then extracted with ethyl acetate (1 L) and the organic phases washed with brine (2 x 100 mL) and dried over magnesium sulphate. The organic phase was filtered and excess solvent removed by rotary evaporation under reduced pressure to afford the product **43** in quantitative yield as a yellow solid. LC/MS (3.93 min (ES-) *m/z* (relative intensity) 367.74 ($[M - H]^{-}$, 100)); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (s, 1H), 7.24 (s, 1H), 3.93 (s, 3H), 1.34 – 1.22 (m, 3H), 1.10 (m, 18H).

*(c) ((2S,4R)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-hydroxypyrrolidin-1-yl)(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)phenyl)methanone (**45**)*

[0502] DCC (29.2 g, 141 mmol, 1.2 eq) was added to a solution of acid **3** (43.5 g, 117.8 mmol, 1eq), and hydroxybenzotriazole hydrate (19.8 g, 129.6 mmol, 1.1 eq) in dichloromethane (200 mL) at 0 °C. The cold bath was removed and the reaction was allowed to proceed for 30 mins at room temperature, at which time a solution of (*2S,4R*)-2-*t*-butyldimethylsilyloxymethyl-4-hydroxypyrrolidine **44** (30 g, 129.6 mmol, 1.1 eq) and triethylamine (24.66 mL, 176 mmol, 1.5

eq) in dichloromethane (100 mL) was added rapidly at -10 °C under argon (on large scale, the addition time could be shortened by cooling the reaction mixture even further. The reaction mixture was allowed to stir at room temperature for 40 minutes to 1 hour and monitored by LC/MS and TLC (EtOAc). The solids were removed by filtration over celite and the organic phase was washed with cold aqueous 0.1 M HCl until the pH was measured at 4 or 5. The organic phase was then washed with water, followed by saturated aqueous sodium bicarbonate and brine. The organic layer was dried over magnesium sulphate, filtered and excess solvent removed by rotary evaporation under reduced pressure. The residue was subjected to column flash chromatography (silica gel; gradient 40/60 ethyl acetate/hexane to 80/20 ethyl acetate/ hexane). Excess solvent was removed by rotary evaporation under reduced pressure afforded the pure product **45**, (45.5 g of pure product 66%, and 17 g of slightly impure product, 90% in total). LC/MS 4.43 min (ES+) *m/z* (relative intensity) 582.92 ($[M + H]^{+\cdot}$, 100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H), 6.74 (s, 1H), 4.54 (s, 1H), 4.40 (s, 1H), 4.13 (s, 1H), 3.86 (s, 3H), 3.77 (d, J = 9.2 Hz, 1H), 3.36 (dd, J = 11.3, 4.5 Hz, 1H), 3.14 – 3.02 (m, 1H), 2.38 – 2.28 (m, 1H), 2.10 (ddd, J = 13.3, 8.4, 2.2 Hz, 1H), 1.36 – 1.19 (m, 3H), 1.15 – 1.05 (m, 18H), 0.91 (s, 9H), 0.17 – 0.05 (m, 6H), (presence of rotamers).

*(d) (S)-5-(((tert-butyldimethylsilyl)oxy)methyl)-1-(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)benzoyl)pyrrolidin-3-one (**46**)*

[0503] TCCA (8.82 g, 40 mmol, 0.7 eq) was added to a stirred solution of **45** (31.7 g, 54 mmol, 1 eq) and TEMPO (0.85 g, 5.4 mmol, 0.1 eq) in dry dichloromethane (250 mL) at 0 °C. The reaction mixture was vigorously stirred for 20 minutess, at which point TLC (50/50 ethyl acetate/hexane) revealed complete consumption of the starting material. The reaction mixture was filtered through celite and the filtrate washed with aqueous saturated sodium bicarbonate (100 mL), sodium thiosulphate (9 g in 300 mL), brine (100 mL) and dried over magnesium

sulphate. Rotary evaporation under reduced pressure afforded product **46** in quantitative yield. LC/MS 4.52 min (ES+) *m/z* (relative intensity) 581.08 ($[M + H]^{+\cdot}$, 100);

[0504] ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 – 7.60 (m, 1H), 6.85 – 6.62 (m, 1H), 4.94 (dd, *J* = 30.8, 7.8 Hz, 1H), 4.50 – 4.16 (m, 1H), 3.99 – 3.82 (m, 3H), 3.80 – 3.34 (m, 3H), 2.92 – 2.17 (m, 2H), 1.40 – 1.18 (m, 3H), 1.11 (t, *J* = 6.2 Hz, 18H), 0.97 – 0.75 (m, 9H), 0.15 – -0.06 (m, 6H), (presence of rotamers).

*(e) (S)-5-(((tert-butyldimethylsilyl)oxy)methyl)-1-(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)benzoyl)-4,5-dihydro-1H-pyrrol-3-yl trifluoromethanesulfonate (**47**)*

[0505] Triflic anhydride (27.7 mL, 46.4 g, 165 mmol, 3 eq) was injected (temperature controlled) to a vigorously stirred suspension of ketone **46** (31.9 g, 55 mmol, 1 eq) in dry dichloromethane (900 mL) in the presence of 2,6-lutidine (25.6 mL, 23.5 g, 220 mmol, 4 eq, dried over sieves) at -50 °C (acetone/dry ice bath). The reaction mixture was allowed to stir for 1.5 hours when LC/MS, following a mini work-up (water/dichloromethane), revealed the reaction to be complete. Water was added to the still cold reaction mixture and the organic layer was separated and washed with saturated sodium bicarbonate, brine and magnesium sulphate. The organic phase was filtered and excess solvent was removed by rotary evaporation under reduced pressure. The residue was subjected to column flash chromatography (silica gel; 10/90 v/v ethyl acetate/hexane), removal of excess eluent afforded the product **47** (37.6 g, 96 %) LC/MS, method 2, 4.32 min (ES+) *m/z* (relative intensity) 712.89 ($[M + H]^{+\cdot}$, 100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (s, 1H), 6.75 (s, 1H), 6.05 (d, *J* = 1.8 Hz, 1H), 4.78 (dd, *J* = 9.8, 5.5 Hz, 1H), 4.15 – 3.75 (m, 5H), 3.17 (ddd, *J* = 16.2, 10.4, 2.3 Hz, 1H), 2.99 (ddd, *J* = 16.3, 4.0, 1.6 Hz, 1H), 1.45 – 1.19 (m, 3H), 1.15 – 1.08 (m, 18H), 1.05 (s, 6H), 0.95 – 0.87 (m, 9H), 0.15 – 0.08 (m, 6H).

*(f) (S)-(2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)(5-methoxy-2-nitro-4-((triisopropylsilyl)oxy)phenyl)methanone (**48**)*

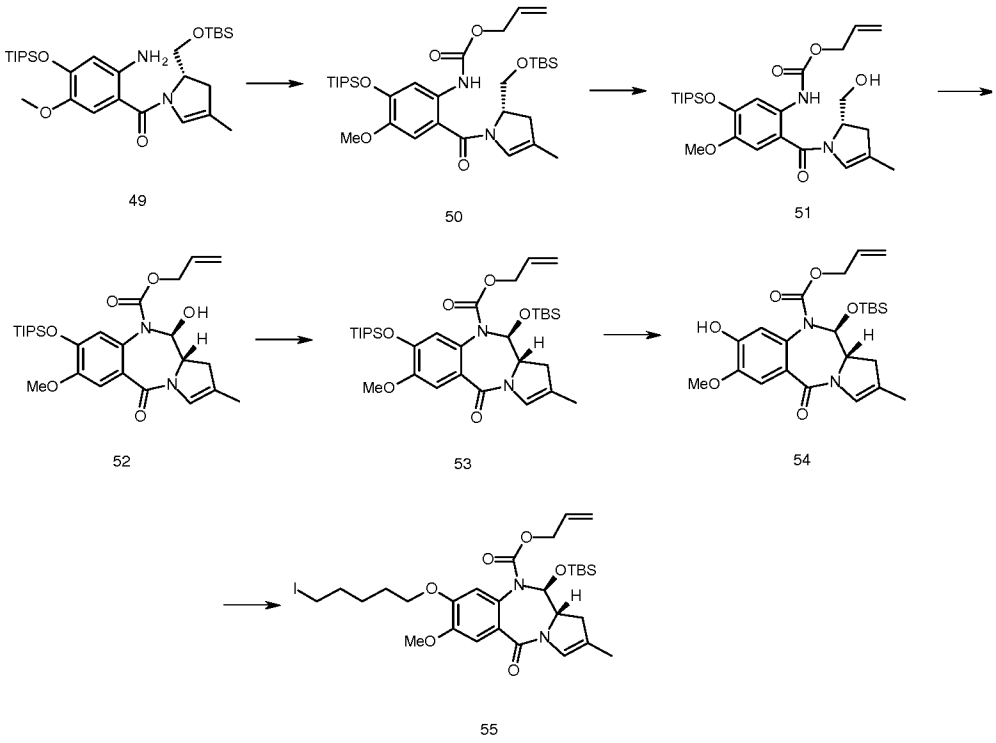
[0506] Triphenylarsine (1.71 g, 5.60 mmol, 0.4 eq) was added to a mixture of triflate **47** (10.00 g, 14 mmol, 1eq), methylboronic acid (2.94 g, 49.1 mmol, 3.5 eq), silver oxide (13 g, 56 mmol, 4 eq) and potassium phosphate tribasic (17.8 g, 84 mmol, 6 eq) in dry dioxane (80 mL) under an argon atmosphere. The reaction was flushed with argon 3 times and bis(benzonitrile)palladium(II) chloride (540 mg, 1.40 mmol, 0.1 eq) was added. The reaction was flushed with argon 3 more times before being warmed instantaneously to 110°C (the drysyn heating block was previously warmed to 110°C prior addition of the flask). After 10 mins the reaction was cooled to room temperature and filtered through a pad celite. The solvent was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to column flash chromatography (silica gel; 10 % ethyl acetate / hexane). Pure fractions were collected and combined, and excess eluent was removed by rotary evaporation under reduced pressure afforded the product **48** (4.5 g, 55 %). LC/MS, 4.27 min (ES+) *m/z* (relative intensity) 579.18 ($[M + H]^{+\cdot}$, 100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (s, 1H), 6.77 (s, 1H), 5.51 (d, *J* = 1.7 Hz, 1H), 4.77 – 4.59 (m, 1H), 3.89 (s, 3H), 2.92 – 2.65 (m, 1H), 2.55 (d, *J* = 14.8 Hz, 1H), 1.62 (d, *J* = 1.1 Hz, 3H), 1.40 – 1.18 (m, 3H), 1.11 (s, 9H), 1.10 (s, 9H), 0.90 (s, 9H), 0.11 (d, *J* = 2.3 Hz, 6H).

*(g) (S)-(2-amino-5-methoxy-4-((triisopropylsilyl)oxy)phenyl)(2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone (**49**)*

[0507] Zinc powder (28 g, 430 mmol, 37 eq) was added to a solution of compound **48** (6.7 g, 11.58 mmol) in 5% formic acid in ethanol v/v (70 mL) at around 15°C. The resulting exotherm was controlled using an ice bath to maintain the temperature of the reaction mixture below 30°C. After 30 minutes the reaction mixture was filtered through a pad of celite. The

filtrate was diluted with ethyl acetate and the organic phase was washed with water, saturated aqueous sodium bicarbonate and brine. The organic phase was dried over magnesium sulphate, filtered and excess solvent removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; 10 % ethyl acetate in hexane). The pure fractions were collected and combined and excess solvent was removed by rotary evaporation under reduced pressure to afford the product **49** (5.1 g, 80 %). LC/MS, 4.23 min (ES+) *m/z* (relative intensity) 550.21 ($[M + H]^{+\cdot}$, 100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (s, 1H), 6.67 (s, 1H), 6.19 (s, 1H), 4.64 – 4.53 (m, *J* = 4.1 Hz, 1H), 4.17 (s, 1H), 3.87 (s, 1H), 3.77 – 3.69 (m, 1H), 3.66 (s, 3H), 2.71 – 2.60 (m, 1H), 2.53 – 2.43 (m, 1H), 2.04 – 1.97 (m, *J* = 11.9 Hz, 1H), 1.62 (s, 3H), 1.26 – 1.13 (m, 3H), 1.08 – 0.99 (m, 18H), 0.82 (s, 9H), 0.03 – -0.03 (m, *J* = 6.2 Hz, 6H).

(ii) (11S,11aS)-allyl 11-((tert-butyldimethylsilyl)oxy)-8-((5-iodopentyl)oxy)-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate



*(a) (S)-allyl (2-(2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamate (**50**)*

[0508] Allyl chloroformate (0.30 mL, 3.00 mmol, 1.1 eq) was added to a solution of amine **49** (1.5 g, 2.73 mmol) in the presence of dry pyridine (0.48 mL, 6.00 mmol, 2.2 eq) in dry dichloromethane (20 mL) at -78°C (acetone/dry ice bath). After 30 minutes, the bath was removed and the reaction mixture was allowed to warm to room temperature. The reaction mixture was diluted with dichloromethane and saturated aqueous copper sulphate was added. The organic layer was then washed sequentially with saturated aqueous sodium bicarbonate and brine. The organic phase was dried over magnesium sulphate, filtered and excess solvent removed by rotary evaporation under reduced pressure to afford the product **50** which was used directly in the next reaction. LC/MS, 4.45 min (ES+) *m/z* (relative intensity) 632.91 ($[M + H]^{+\cdot}$, 100)

*(b) (S)-allyl (2-(2-(hydroxymethyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamate (**51**)*

[0509] The crude **50** was dissolved in a 7:1:1:2 mixture of acetic acid/methanol/tetrahydrofuran/water (28:4:4:8 mL) and allowed to stir at room temperature. After 3 hours, complete disappearance of starting material was observed by LC/MS. The reaction mixture was diluted with ethyl acetate and washed sequentially with water (2 x 500 mL), saturated aqueous sodium bicarbonate (200 mL) and brine. The organic phase was dried over magnesium sulphate filtered and excess ethyl acetate removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel, 25% ethyl acetate in hexane). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to afford the desired product **51** (1 g, 71 %). LC/MS, 3.70 min (ES+) *m/z* (relative intensity) 519.13 ($[M + H]^{+\cdot}$, 95); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.69 (s, 1H), 6.78 (s, 1H), 6.15 (s, 1H), 5.95 (ddt, *J* = 17.2, 10.5, 5.7 Hz, 1H), 5.33 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.23 (ddd, *J* = 10.4, 2.6, 1.3 Hz, 1H), 4.73 (tt, *J* = 7.8, 4.8 Hz, 1H), 4.63 (dt, *J* = 5.7, 1.4 Hz, 2H), 4.54 (s, 1H), 3.89 – 3.70 (m, 5H), 2.87 (dd, *J* = 16.5, 10.5 Hz, 1H), 2.19 (dd, *J* = 16.8, 4.6 Hz, 1H), 1.70 (d, *J* = 1.3 Hz, 3H), 1.38 – 1.23 (m, 3H), 1.12 (s, 10H), 1.10 (s, 8H).

*(c) (11S,11aS)-allyl 11-hydroxy-7-methoxy-2-methyl-5-oxo-8-((triisopropylsilyl)oxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**52**)*

[0510] Dimethyl sulphoxide (0.35 mL, 4.83 mmol, 2.5 eq) was added dropwise to a solution of oxalyl chloride (0.2 mL, 2.32 mmol, 1.2 eq) in dry dichloromethane (10 mL) at -78°C (dry ice /acetone bath) under an atmosphere of argon. After 10 minutes a solution of **51** (1 g, 1.93 mmol) in dry dichloromethane (8 mL) was added slowly with the temperature still at -78°C. After 15 min triethylamine (1.35 mL, dried over 4Å molecular sieves, 9.65 mmol, 5 eq) was

added dropwise and the dry ice/acetone bath was removed. The reaction mixture was allowed to reach room temperature and was extracted with cold hydrochloric acid (0.1 M), saturated aqueous sodium bicarbonate and brine. The organic phase was dried over magnesium sulphate, filtered and excess dichloromethane was removed by rotary evaporation under reduced pressure to afford product **52** (658 mg, 66%). LC/MS, 3.52 min (ES+) *m/z* (relative intensity) 517.14 ([*M* + H]$^{+\cdot}$, 100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (s, 1H), 6.75 – 6.63 (m, *J* = 8.8, 4.0 Hz, 2H), 5.89 – 5.64 (m, *J* = 9.6, 4.1 Hz, 2H), 5.23 – 5.03 (m, 2H), 4.68 – 4.38 (m, 2H), 3.84 (s, 3H), 3.83 – 3.77 (m, 1H), 3.40 (s, 1H), 3.05 – 2.83 (m, 1H), 2.59 (d, *J* = 17.1 Hz, 1H), 1.78 (d, *J* = 1.3 Hz, 3H), 1.33 – 1.16 (m, 3H), 1.09 (d, *J* = 2.2 Hz, 9H), 1.07 (d, *J* = 2.1 Hz, 9H).

*(d) (11S,11aS)-allyl 11-((tert-butyldimethylsilyl)oxy)-7-methoxy-2-methyl-5-oxo-8-((triisopropylsilyl)oxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**53**)*

[0511] *Tert*-butyldimethylsilyltriflate (0.70 mL, 3.00 mmol, 3 eq) was added to a solution of compound **52** (520 mg, 1.00 mmol) and 2,6-lutidine (0.46 mL, 4.00 mmol, 4 eq) in dry dichloromethane (40 mL) at 0°C under argon. After 10 min, the cold bath was removed and the reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was extracted with water, saturated aqueous sodium bicarbonate and brine. The organic phase was dried over magnesium sulphate, filtered and excess was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; gradient, 10 % ethyl acetate in hexane to 20 % ethyl acetate in hexane). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to give the product **53** (540 mg, 85 %). LC/MS, 4.42 min (ES+) *m/z* (relative intensity) 653.14 ([*M* + Na]$^{+\cdot}$, 100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (s, 1H), 6.71 – 6.64 (m, *J* = 5.5 Hz, 2H), 5.83 (d, *J* = 9.0 Hz, 1H), 5.80 – 5.68 (m, *J* = 5.9 Hz, 1H), 5.14 – 5.06 (m, 2H), 4.58 (dd, *J* =

13.2, 5.2 Hz, 1H), 4.36 (dd, *J* = 13.3, 5.5 Hz, 1H), 3.84 (s, 3H), 3.71 (td, *J* = 10.1, 3.8 Hz, 1H), 2.91 (dd, *J* = 16.9, 10.3 Hz, 1H), 2.36 (d, *J* = 16.8 Hz, 1H), 1.75 (s, 3H), 1.31 – 1.16 (m, 3H), 1.12 – 1.01 (m, *J* = 7.4, 2.1 Hz, 18H), 0.89 – 0.81 (m, 9H), 0.25 (s, 3H), 0.19 (s, 3H).

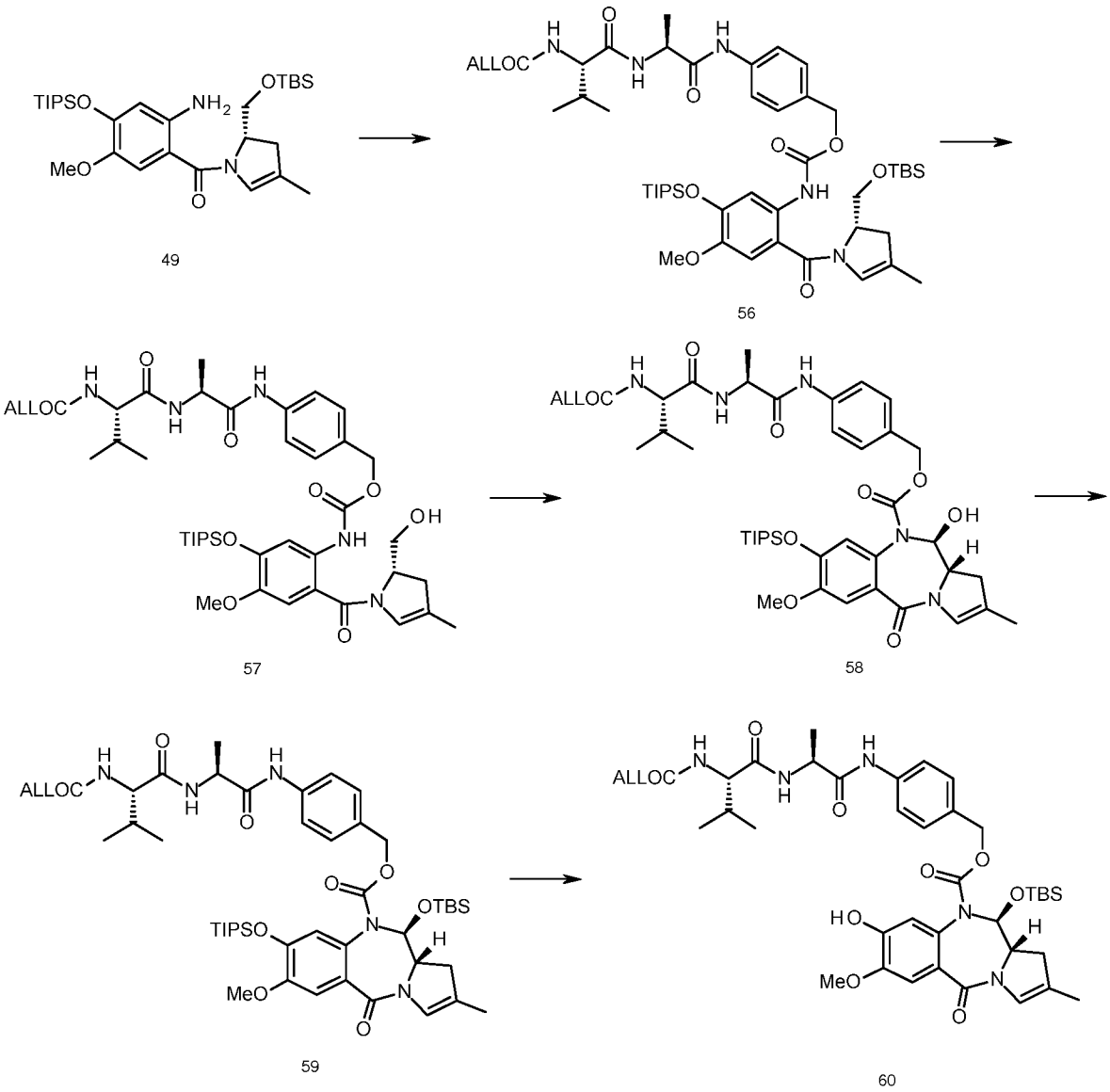
*(e) (11S,11aS)-allyl 11-((tert-butyldimethylsilyl)oxy)-8-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**54**)*

[0512] Lithium acetate (87 mg, 0.85 mmol) was added to a solution of compound **53** (540 mg, 0.85 mmol) in wet dimethylformamide (6 mL, 50:1 DMF/water). After 4 hours, the reaction was complete and the reaction mixture was diluted with ethyl acetate (25 mL) and washed with aqueous citric acid solution (pH ~ 3), water and brine. The organic layer was dried over magnesium sulphate filtered and excess ethyl acetate was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; gradient, 25% to 75% ethyl acetate in hexane). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to give the product **54** (400 mg, quantitative). LC/MS, (3.33 min (ES+) *m/z* (relative intensity) 475.26 ($[M+H]^+$, 100).

*(f) (11S,11aS)-allyl 11-((tert-butyldimethylsilyl)oxy)-8-((5-iodopentyl)oxy)-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**55**)*

[0513] Diiodopentane (0.63 mL, 4.21 mmol, 5 eq) and potassium carbonate (116 mg, 0.84 mmol, 1 eq) were added to a solution of phenol **54** (400 mg, 0.84 mmol) in acetone (4 mL, dried over molecular sieves). The reaction mixture was then warmed to 60°C and stirred for 6 hours. Acetone was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; 50/50, v/v, hexane/ethyl acetate,). Pure fractions were collected and combined and excess eluent was removed to provide **55** in 90% yield. LC/MS, 3.90 min (ES+) *m/z* (relative intensity) 670.91 ($[M]^+$, 100). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 (s, 1H), 6.69 (s, 1H), 6.60 (s, 1H), 5.87 (d, *J* = 8.8 Hz, 1H), 5.83 – 5.68 (m, *J* = 5.6 Hz, 1H), 5.15 – 5.01 (m, 2H), 4.67 – 4.58 (m, 1H), 4.45 – 4.35 (m, 1H), 4.04 – 3.93 (m, 2H), 3.91 (s, 3H), 3.73 (td, *J* = 10.0, 3.8 Hz, 1H), 3.25 – 3.14 (m, *J* = 8.5, 7.0 Hz, 2H), 2.92 (dd, *J* = 16.8, 10.3 Hz, 1H), 2.38 (d, *J* = 16.8 Hz, 1H), 1.95 – 1.81 (m, 4H), 1.77 (s, 3H), 1.64 – 1.49 (m, 2H), 0.88 (s, 9H), 0.25 (s, 3H), 0.23 (s, 3H).

(iii) (11S,11aS)-4-(2-(1-((1-(allyloxy)-4-methyl-1,2-dioxopentan-3-yl)amino)-1-oxopropan-2-yl)hydrazinyl)benzyl 11-((tert-butyldimethylsilyl)oxy)-8-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (70)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OTBS
MeO
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N
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ALLOC
H
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O
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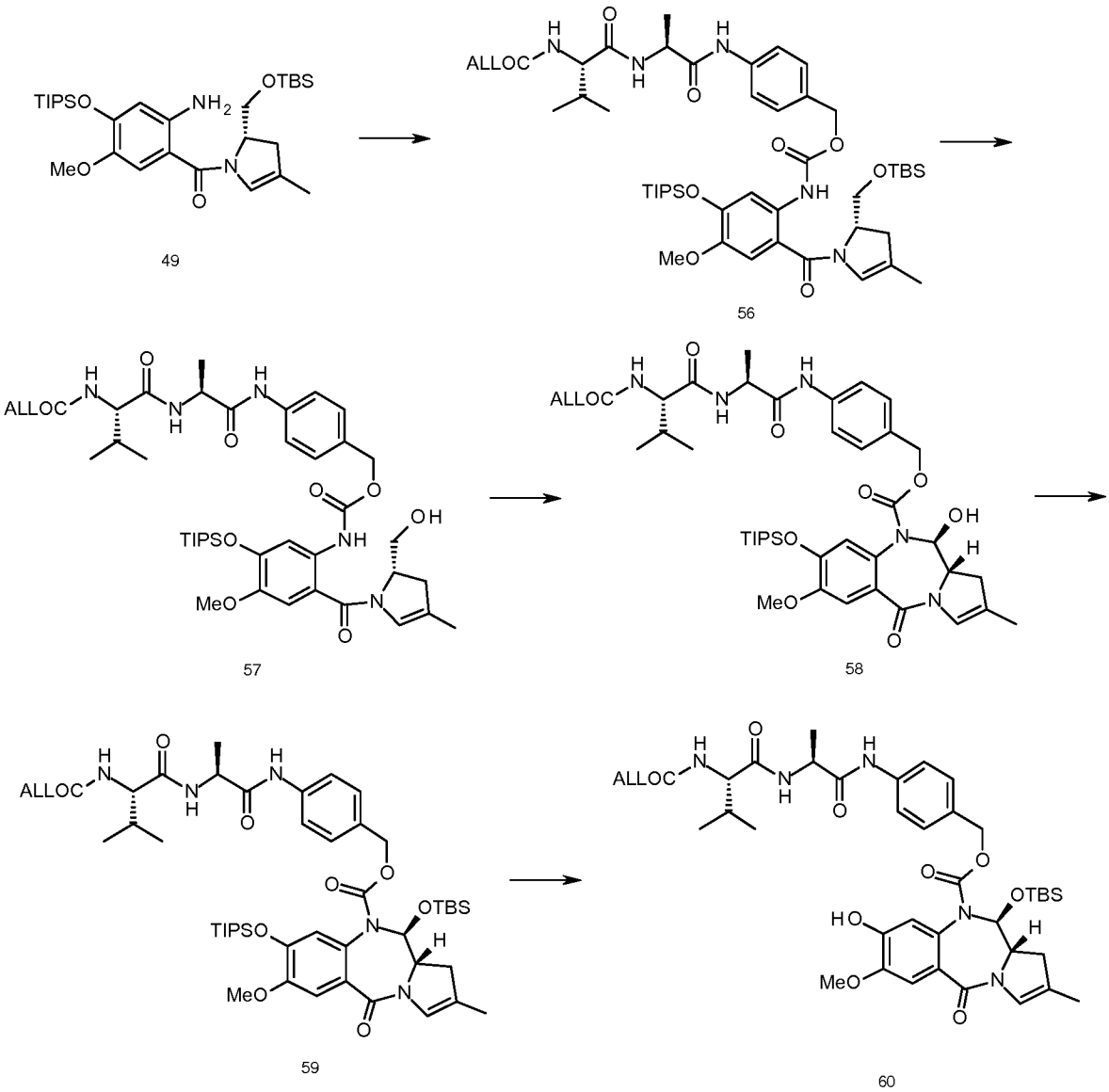
HO



*(a) Allyl 3-(2-(2-(4-((((2-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamoyl)oxy)methyl)phenyl)hydrazinyl)propanamido)-4-methyl-2-oxopentanoate (**56**)*

[0514] Triethylamine (2.23 mL, 18.04 mmol, 2.2 eq) was added to a stirred solution of the amine **49** (4 g, 8.20 mmol) and triphosgene (778 mg, 2.95 mmol, 0.36 eq) in dry tetrahydrofuran (40 mL) at 5 °C (ice bath). The progress of the isocyanate reaction was

monitored by periodically removing aliquots from the reaction mixture and quenching with methanol and performing LC/MS analysis. Once the isocyanate formation was complete a solution of the alloc-Val-Ala-PABOH (4.12 g, 12.30 mmol, 1.5 eq) and triethylamine (1.52 mL, 12.30 mmol, 1.5 eq) in dry tetrahydrofuran (40 mL) was rapidly added by injection to the freshly prepared isocyanate. The reaction mixture was allowed to stir at 40 °C for 4 hours. Excess solvent was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; gradient, 1 % methanol to 5% methanol in dichloromethane). (Alternative chromatography conditions using EtOAc and Hexane have also been successful). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to give the product **56** (3.9 g, 50%). LC/MS, 4.23 min (ES+) *m/z* (relative intensity) 952.36 ($[M + H]^{+\cdot}$, 100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (br s, 1H), 8.46 (s, 1H), 7.77 (br s, 1H), 7.53 (d, *J* = 8.4 Hz, 2H), 7.32 (d, *J* = 8.5 Hz, 2H), 6.76 (s, 1H), 6.57 (d, *J* = 7.6 Hz, 1H), 6.17 (s, 1H), 6.03 – 5.83 (m, 1H), 5.26 (dd, *J* = 33.8, 13.5 Hz, 3H), 5.10 (s, 2H), 4.70 – 4.60 (m, 2H), 4.58 (dd, *J* = 5.7, 1.3 Hz, 2H), 4.06 – 3.99 (m, 1H), 3.92 (s, 1H), 3.82 – 3.71 (m, 1H), 3.75 (s, 3H), 2.79 – 2.64 (m, 1H), 2.54 (d, *J* = 12.9 Hz, 1H), 2.16 (dq, *J* = 13.5, 6.7 Hz, 1H), 1.67 (s, 3H), 1.46 (d, *J* = 7.0 Hz, 3H), 1.35 – 1.24 (m, 3H), 1.12 (s, 9H), 1.10 (s, 9H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.87 (s, 9H), 0.07 – -0.02 (m, 6H).

*(b) Allyl 3-(2-(2-(4-((((2-((S)-2-(hydroxymethyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamoyl)oxy)methyl)phenyl)hydrazinyl)propanamido)-4-methyl-2-oxopentanoate (**57**)*

[0515] The TBS ether **56** (1.32 g, 1.38 mmol) was dissolved in a 7:1:1:2 mixture of acetic acid/methanol/tetrahydrofuran/water (14:2:2:4 mL) and allowed to stir at room temperature. After 3 hours no more starting material was observed by LC/MS. The reaction

mixture was diluted with ethyl acetate (25 mL) and washed sequentially with water, saturated aqueous sodium bicarbonate and brine. The organic phase was dried over magnesium sulphate filtered and excess ethyl acetate removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel, 2% methanol in dichloromethane). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to afford the desired product **57** (920 mg, 80%). LC/MS, 3.60 min (ES+) *m/z* (relative intensity) 838.18 ($[M+H]^{+\cdot}$, 100). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (s, 1H), 8.35 (s, 1H), 7.68 (s, 1H), 7.52 (d, *J* = 8.1 Hz, 2H), 7.31 (d, *J* = 8.4 Hz, 2H), 6.77 (s, 1H), 6.71 (d, *J* = 7.5 Hz, 1H), 6.13 (s, 1H), 5.97 – 5.82 (m, *J* = 5.7 Hz, 1H), 5.41 – 5.15 (m, 3H), 5.10 (d, *J* = 3.5 Hz, 2H), 4.76 – 4.42 (m, 5H), 4.03 (t, *J* = 6.6 Hz, 1H), 3.77 (s, 5H), 2.84 (dd, *J* = 16.7, 10.4 Hz, 1H), 2.26 – 2.08 (m, 2H), 1.68 (s, 3H), 1.44 (d, *J* = 7.0 Hz, 3H), 1.30 (dt, *J* = 14.7, 7.4 Hz, 3H), 1.12 (s, 9H), 1.10 (s, 9H), 0.96 (d, *J* = 6.8 Hz, 3H), 0.93 (d, *J* = 6.8 Hz, 3H).

*(c) (11S,11aS)-4-(2-(1-((1-(allyloxy)-4-methyl-1,2-dioxopentan-3-yl)amino)-1-oxopropan-2-yl)hydrazinyl)benzyl 11-hydroxy-7-methoxy-2-methyl-5-oxo-8-((triisopropylsilyl)oxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**58**)*

[0516] Dimethyl sulphoxide (0.2 mL, 2.75 mmol, 2.5 eq) was added dropwise to a solution of oxalyl chloride (0.11 mL, 1.32 mmol, 1.2 eq) in dry dichloromethane (7 mL) at -78°C (dry ice /acetone bath) under an atmosphere of argon. After 10 minutes a solution of **57** (920 mg, 1.10 mmol) in dry dichloromethane (5 mL) was added slowly with the temperature still at -78°C. After 15 min triethylamine (0.77 mL, dried over 4Å molecular sieves, 5.50 mmol, 5 eq) was added dropwise and the dry ice/acetone bath was removed. The reaction mixture was allowed to reach room temperature and was extracted with cold hydrochloric acid (0.1 M), saturated

aqueous sodium bicarbonate and brine. The organic phase was dried over magnesium sulphate, filtered and excess dichloromethane was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to column flash chromatography (silica gel; gradient 2% methanol to 5 % methanol in dichloromethane). Pure fractions were collected and combined and removal of excess eluent by rotary evaporation under reduced pressure afforded the product **58** (550 mg, 60%). LC/MS, 3.43 min (ES+) *m/z* (relative intensity) 836.01 ($[M]^{+\cdot}$, 100). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.52 – 7.40 (m, 2H), 7.21 – 7.08 (m, *J* = 11.5 Hz, 2H), 6.67 (s, 1H), 6.60 – 6.47 (m, *J* = 7.4 Hz, 1H), 5.97 – 5.83 (m, 1H), 5.79 – 5.66 (m, 1H), 5.38 – 4.90 (m, 6H), 4.68 – 4.52 (m, *J* = 18.4, 5.5 Hz, 4H), 4.04 – 3.94 (m, *J* = 6.5 Hz, 1H), 3.87 – 3.76 (m, 5H), 3.00 – 2.88 (m, 1H), 2.66 – 2.49 (m, 2H), 2.21 – 2.08 (m, 2H), 1.76 (s, 3H), 1.45 (d, *J* = 7.0 Hz, 3H), 1.09 – 0.98 (m, *J* = 8.9 Hz, 18H), 0.96 (d, *J* = 6.7 Hz, 3H), 0.93 (d, *J* = 6.9 Hz, 3H).

*(d) (11S,11aS)-4-(2-(1-((1-(Allyloxy)-4-methyl-1,2-dioxopentan-3-yl)amino)-1-oxopropan-2-yl)hydrazinyl)benzyl 11-((tert-butyldimethylsilyl)oxy)-7-methoxy-2-methyl-5-oxo-8-((triisopropylsilyl)oxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**59**)*

[0517] *Tert*-butyldimethylsilyltriflate (0.38 mL, 1.62 mmol, 3 eq) was added to a solution of compound **58** (450 mg, 0.54 mmol) and 2,6-lutidine (0.25 mL, 2.16 mmol, 4 eq) in dry dichloromethane (5 mL) at 0°C under argon. After 10 min, the cold bath was removed and the reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was extracted with water, saturated aqueous sodium bicarbonate and brine. The organic phase was dried over magnesium sulphate, filtered and excess solvent was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to column flash chromatography (silica gel; 50/50 v/v hexane/ethyl acetate). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to give the product **59** (334

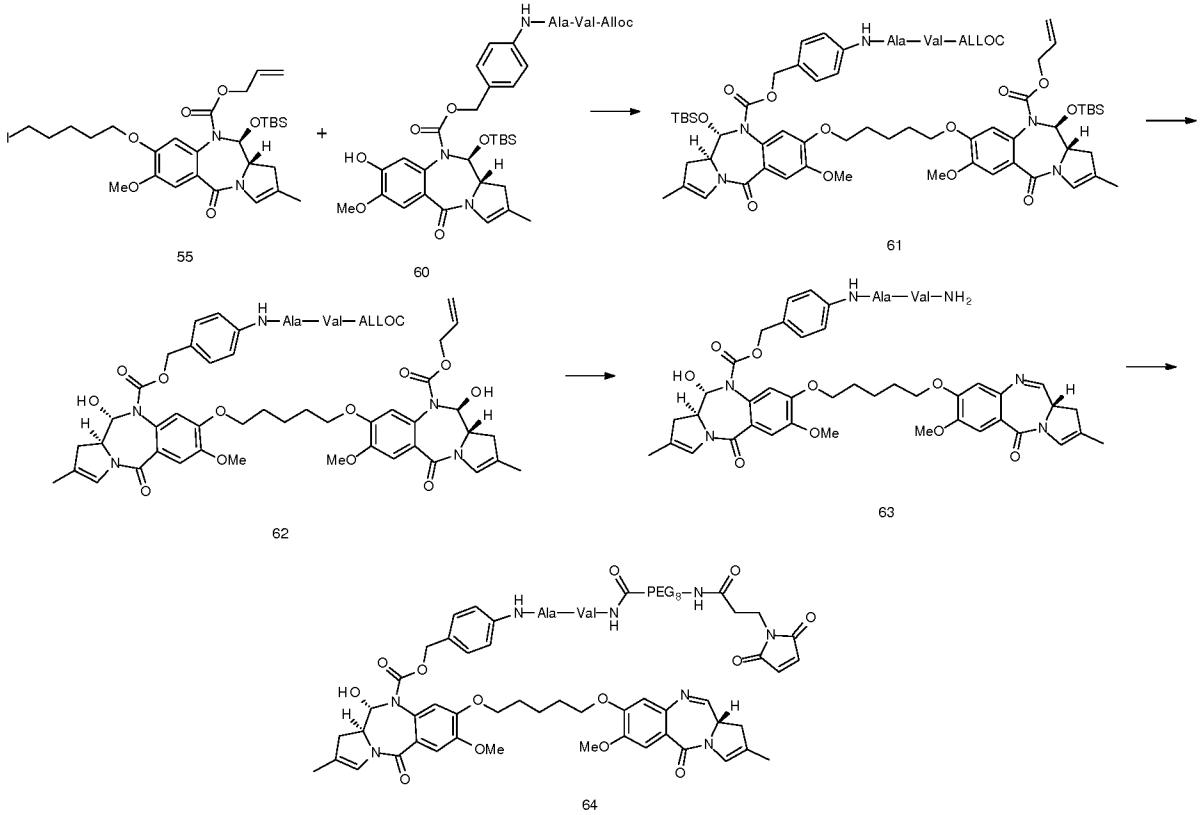
mg, 65%). LC/MS, 4.18 min (ES+) *m/z* (relative intensity) 950.50 ($[M]^{+\cdot}$, 100). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (s, 1H), 8.02 (s, 1H), 7.44 (d, *J* = 7.6 Hz, 2H), 7.21 (s, 1H), 7.08 (d, *J* = 8.2 Hz, 2H), 6.72 – 6.61 (m, *J* = 8.9 Hz, 2H), 6.16 (s, 1H), 5.97 – 5.79 (m, *J* = 24.4, 7.5 Hz, 2H), 5.41 – 5.08 (m, 5H), 4.86 (d, *J* = 12.5 Hz, 1H), 4.69 – 4.60 (m, 1H), 4.57 (s, 1H), 4.03 (t, *J* = 6.7 Hz, 1H), 3.87 (s, 3H), 3.74 (td, *J* = 9.6, 3.6 Hz, 1H), 2.43 – 2.09 (m, *J* = 34.8, 19.4, 11.7 Hz, 3H), 1.76 (s, 3H), 1.43 (d, *J* = 6.9 Hz, 3H), 1.30 – 1.21 (m, 3H), 0.97 (d, *J* = 6.7 Hz, 3H), 0.92 (t, *J* = 8.4 Hz, 3H), 0.84 (s, 9H), 0.23 (s, 3H), 0.12 (s, 3H).

*(e) (11S,11aS)-4-(2-(1-((1-(Allyloxy)-4-methyl-1,2-dioxopentan-3-yl)amino)-1-oxopropan-2-yl)hydrazinyl)benzyl 11-((tert-butyldimethylsilyl)oxy)-8-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**60**)*

[0518] Lithium acetate (50 mg, 0.49 mmol) was added to a solution of compound **59** (470 mg, 0.49 mmol) in wet dimethylformamide (4 mL, 50:1 DMF/water). After 4 hours, the reaction was complete and the reaction mixture was diluted with ethyl acetate and washed with citric acid (pH ~ 3), water and brine. The organic layer was dried over magnesium sulphate filtered and excess ethyl acetate was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to column flash chromatography (silica gel; gradient, 50/50 to 25/75 v/v hexane/ethyl acetate). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to give the product **60** (400 mg, quantitative). LC/MS, 3.32 min (ES+) *m/z* (relative intensity) 794.18 ($[M+H]^{+\cdot}$, 100). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (s, 1H), 8.02 (s, 1H), 7.44 (d, *J* = 7.6 Hz, 2H), 7.21 (s, 1H), 7.08 (d, *J* = 8.2 Hz, 2H), 6.72 – 6.61 (m, *J* = 8.9 Hz, 2H), 6.16 (s, 1H), 5.97 – 5.79 (m, *J* = 24.4, 7.5 Hz, 2H), 5.41 – 5.08 (m, 5H), 4.86 (d, *J* = 12.5 Hz, 1H), 4.69 – 4.60 (m, 1H), 4.57 (s, 1H), 4.03 (t, *J* = 6.7 Hz, 1H), 3.87 (s, 3H), 3.74 (td, *J* = 9.6, 3.6 Hz, 1H), 2.43 – 2.09 (m, *J* = 34.8, 19.4, 11.7

Hz, 3H), 1.76 (s, 3H), 1.43 (d, *J* = 6.9 Hz, 3H), 1.30 – 1.21 (m, 3H), 0.97 (d, *J* = 6.7 Hz, 3H), 0.92 (t, *J* = 8.4 Hz, 3H), 0.84 (s, 9H), 0.23 (s, 3H), 0.12 (s, 3H).

(iv) (11S,11aS)-4-((2S,5S)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl 11-hydroxy-7-methoxy-8-((5-(((S)-7-methoxy-2-methyl-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)pentyl)oxy)-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (64)



*(a) (11S)-allyl 8-((5-(((11S)-10-(((4-(2-(1-((1-(allyloxy)-4-methyl-1,2-dioxopentan-3-yl)amino)-1-oxopropan-2-yl)hydrazinyl)benzyl)oxy)carbonyl)-11-((tert-butyldimethylsilyl)oxy)-7-methoxy-2-methyl-5-oxo-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)pentyl)oxy)-11-((tert-butyldimethylsilyl)oxy)-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**61**)*

[0519] Potassium carbonate (70 mg, 0.504 mmol, 1 eq) was added to a solution of **55** (370 mg, 0.552 mmol, 1.2 eq) and phenol **60** (400 mg, 0.504 mmol) in dry acetone (25 mL). The

reaction was stirred 8 hours at 70°C. The LC/MS showed that all the starting material was not consumed, so the reaction was allowed to stir overnight at room temperature and stirred for an additional 2 hours the next day. Acetone was removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; 80% ethyl acetate in hexane to 100% ethyl acetate). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to give the product **61** (385 mg, 57%). LC/MS, 4.07 min (ES+) *m/z* (relative intensity) 1336.55 ($[M+H]^{+\cdot}$, 50).

*(b) (11S)-allyl 8-((5-(((11S)-10-(((4-(2-(1-((1-(allyloxy)-4-methyl-1,2-dioxopentan-3-yl)amino)-1-oxopropan-2-yl)hydrazinyl)benzyl)oxy)carbonyl)-11-hydroxy-7-methoxy-2-methyl-5-oxo-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)pentyl)oxy)-11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**62**)*

[0520] Tetra-*n*-butylammonium fluoride (1M, 0.34 mL, 0.34 mmol, 2 eq) was added to a solution of **61** (230 mg, 0.172 mmol) in dry tetrahydrofuran (3 mL). The starting material was totally consumed after 10 minutes. The reaction mixture was diluted with ethyl acetate (30 mL) and washed sequentially with water and brine. The organic phase was dried over magnesium sulphate filtered and excess ethyl acetate removed by rotary evaporation under reduced pressure. The resulting residue **62** was used as a crude mixture for the next reaction. LC/MS, 2.87 min (ES+) *m/z* (relative intensity) 1108.11 ($[M+H]^{+\cdot}$, 100).

*(c) (11S)-4-(2-(1-((1-amino-3-methyl-1-oxobutan-2-yl)amino)-1-oxopropan-2-yl)hydrazinyl)benzyl 11-hydroxy-7-methoxy-8-((5-((7-methoxy-2-methyl-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)pentyl)oxy)-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**63**)*

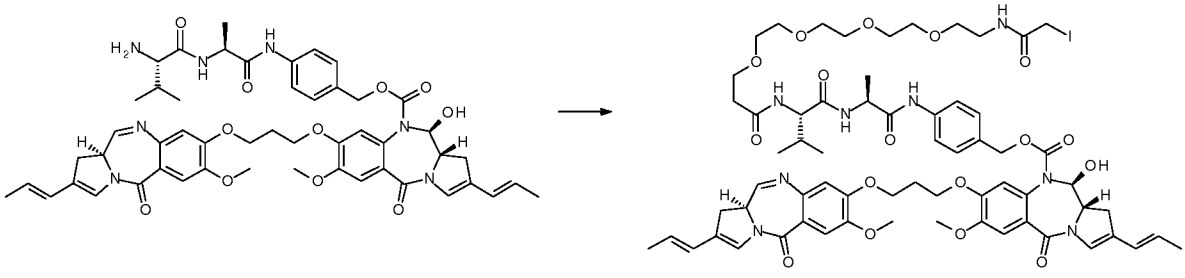
[0521] *Tetrakis*(triphenylphosphine)palladium(0) (12 mg, 0.01 mmol, 0.06 eq) was added to a solution of crude **62** (0.172 mmol) and pyrrolidine (36 µL, 0.43 mmol, 2.5 eq) in dry

dichloromethane (10 mL). The reaction mixture was stirred 20 minutes and diluted with dichloromethane and washed sequentially with saturated aqueous ammonium chloride and brine. The organic phase was dried over magnesium sulphate filtered and excess dichloromethane removed by rotary evaporation under reduced pressure. The resulting residue **63** was used as a crude mixture for the next reaction. LC/MS, 2.38 min (ES+) *m/z* (relative intensity) 922.16 ($[M+H]^{+\cdot}$, 40).

*(d) (11S,11aS)-4-((2S,5S)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl 11-hydroxy-7-methoxy-8-((5-(((S)-7-methoxy-2-methyl-5-oxo-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)pentyl)oxy)-2-methyl-5-oxo-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**64**)*

[0522] 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI, 33 mg, 0.172 mmol) was added to a solution of crude **63** (0.172 mmol) and Mal-$(PEG)_8$-acid (100 mg, 0.172 mmol) in dry dichloromethane (10 mL). The reaction was stirred for 2 hours and the presence ofstarting material was no longer observed by LC/MS. The reaction was diluted with dichloromethane and washed sequentially with water and brine. The organic phase was dried over magnesium sulphate filtered and excess dichloromethane removed by rotary evaporation under reduced pressure. The resulting residue was subjected to flash column chromatography (silica gel; 100% chloroform to 10% methanol in chloroform). Pure fractions were collected and combined and excess eluent was removed by rotary evaporation under reduced pressure to give **64 (E)** (60 mg, 25% over 3 steps).

Example 18



65 66

Compound 65 is compound 79 of WO 2011/130598

[0523] (11S)-4-(1-iodo-20-isopropyl-23-methyl-2,18,21-trioxo-6,9,12,15-tetraoxa-3,19,22-triazatetracosanamido)benzyl 11-hydroxy-7-methoxy-8-(3-((7-methoxy-5-oxo-2-((E)-prop-1-en-1-yl)-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yl)oxy)propoxy)-5-oxo-2-((E)-prop-1-en-1-yl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**66**)

[0524] *N,N'*-diisopropylcarbodiimide (DIC, 4.71 µL, 0.0304 mmol) was added to a solution of amine **65** (0.0276 mmol) and Iodo-$(PEG)_4$-acid (13.1 mg, 0.0304 mmol) in dry dichloromethane (0.8 mL). The reaction was stirred for 3 hours and the presence of starting material was no longer observed by LC/MS. The reaction mixture was directly loaded onto a thin-layer chromatography (TLC) plate and purified by prep-TLC (10% methanol in chloroform). Pure bands were scraped off the TLC plate, taken up in 10% methanol in chloroform, filtered and excess eluent removed by rotary evaporation under reduced pressure to give **66 (D)** (20.9 mg, 56%). LC/MS, method 2, 3.08 min (ES+) *m/z* (relative intensity) 1361.16 ($[M+H]^+$, 100).

[0525]

Example 19

General Experimental Methods

[0526] LCMS data were obtained using an Agilent 1200 series LC/MS with an Agilent 6110 quadrupole MS, with Electrospray ionisation. Mobile phase A - 0.1% Acetic acid in water. Mobile Phase B - 0.1% in acetonitrile. Flow rate of 1.00ml/min. Gradient from 5% B rising up to 95% B over 3 minutes, remaining at 95% B for 1 minute and then back down to 5% B over 6 seconds. The total run time is 5 minutes. Column: Phenomenex Gemini-NX 3µm C18, 30 x 2.00mm. Chromatograms based on UV detection at 254nm. Mass Spectra were achieved using the MS in positive mode. Proton NMR chemical shift values were measured on the delta scale at 400 MHz using a Bruker AV400. The following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Coupling constants are reported in Hz. Unless otherwise stated, column chromatography (by the flash procedure) were performed on Merck Kieselgel silica (Art. 9385). Mass spectroscopy (MS) data were collected using a Waters Micromass LCT instrument coupled to a Waters 2795 HPLC separations module. Thin Layer Chromatography (TLC) was performed on silica gel aluminium plates (Merck 60, F_{254}). All other chemicals and solvents were purchased from Sigma-Aldrich or Fisher Scientific and were used as supplied without further purification.

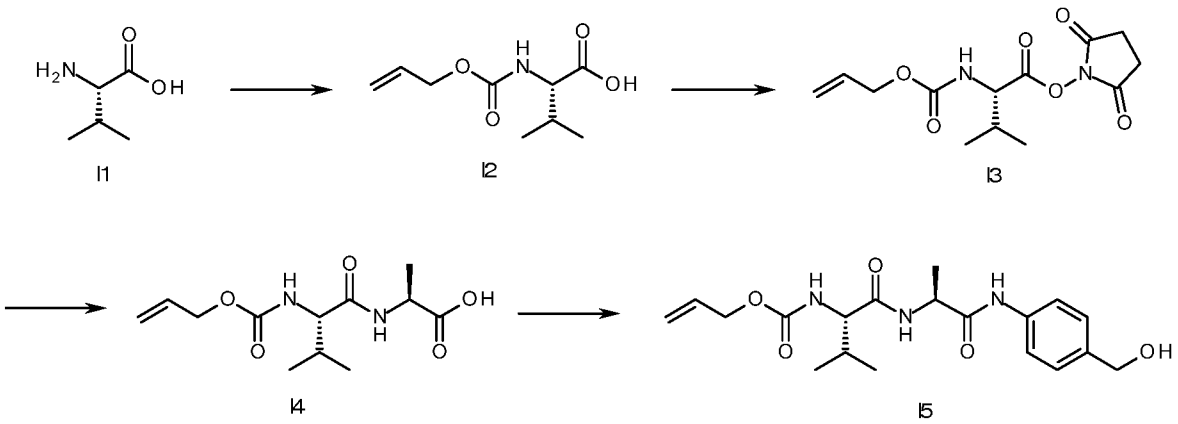
[0527] Optical rotations were measured on an ADP 220 polarimeter (Bellingham Stanley Ltd.) and concentrations (*c*) are given in g/100mL. Melting points were measured using a digital melting point apparatus (Electrothermal). IR spectra were recorded on a Perkin-Elmer Spectrum 1000 FT IR Spectrometer. ^{1}H and ^{13}C NMR spectra were acquired at 300 K using a Bruker Avance NMR spectrometer at 400 and 100 MHz, respectively. Chemical shifts are reported relative to TMS ($\delta = 0.0$ ppm), and signals are designated as s (singlet), d (doublet), t (triplet), dt

(double triplet), dd (doublet of doublets), ddd (double doublet of doublets) or m (multiplet), with coupling constants given in Hertz (Hz). Mass spectroscopy (MS) data were collected using a Waters Micromass ZQ instrument coupled to a Waters 2695 HPLC with a Waters 2996 PDA. Waters Micromass ZQ parameters used were: Capillary (kV), 3.38; Cone (V), 35; Extractor (V), 3.0; Source temperature (°C), 100; Desolvation Temperature (°C), 200; Cone flow rate (L/h), 50; De-solvation flow rate (L/h), 250. High-resolution mass spectroscopy (HRMS) data were recorded on a Waters Micromass QTOF Global in positive W-mode using metal-coated borosilicate glass tips to introduce the samples into the instrument. Thin Layer Chromatography (TLC) was performed on silica gel aluminium plates (Merck 60, F_{254}), and flash chromatography utilised silica gel (Merck 60, 230-400 mesh ASTM). Except for the HOBt (NovaBiochem) and solid-supported reagents (Argonaut), all other chemicals and solvents were purchased from Sigma-Aldrich and were used as supplied without further purification. Anhydrous solvents were prepared by distillation under a dry nitrogen atmosphere in the presence of an appropriate drying agent, and were stored over 4Å molecular sieves or sodium wire. Petroleum ether refers to the fraction boiling at 40-60°C.

[0528] General LC/MS conditions: The HPLC (Waters Alliance 2695) was run using a mobile phase of water (A) (formic acid 0.1%) and acetonitrile (B) (formic acid 0.1%). Gradient: initial composition 5% B over 1.0 min then 5% B to 95% B within 3 min. The composition was held for 0.5 min at 95% B, and then returned to 5% B in 0.3 minutes. Total gradient run time equals 5 min. Flow rate 3.0 mL/min, 400µL was split *via* a zero dead volume tee piece which passes into the mass spectrometer. Wavelength detection range: 220 to 400 nm. Function type: diode array (535 scans). Column: Phenomenex® Onyx Monolithic C18 50 x 4.60 mm

(i) Key Intermediates

(a)



(a-i) (S)-2-(allyloxycarbonylamino)-3-methylbutanoic acid (I2)

[0529] Allyl chloroformate (36.2 ml, 340.59 mmol, 1.2 eq) was added dropwise to a stirred solution of L-valine (**I1**)(33.25 g, 283.82 mmol, 1.0 eq) and potassium carbonate (59.27 g, 425.74 mmol, 1.5 eq) in water (650 mL) and THF (650 mL). The reaction mixture was stirred at room temperature for 18 hours, then the solvent was concentrated under reduced pressure and the remaining solution extracted with diethyl ether (3 x 100 mL). The aqueous portion was acidified to pH 2 with conc. HCl and extracted with DCM (3 x 100 mL). The combined organics were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the product as a colourless oil (57.1 g, assumed 100% yield). LC/MS (1.966 min (ES^+)), *m/z*: 202.1 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 12.57 (br s, 1H), 7.43 (d, 1H, *J* = 8.6 Hz), 5.96 – 5.86 (m, 1H), 5.30 (ddd, 1H, *J* = 17.2, 3.4, 1.7 Hz), 5.18 (ddd, 1H, *J* = 10.4, 2.9, 1.6 Hz), 4.48 (dt, 2H, *J* = 5.3, 1.5 Hz), 3.85 (dd, 1H, *J* = 8.6, 6.0 Hz), 2.03 (oct, 1H, *J* = 6.6 Hz), 0.89 (d, 3H, *J* = 6.4 Hz), 0.87 (d, 3H, *J* = 6.5 Hz).

*(a-ii) (S)-2,5-dioxopyrrolidin-1-yl 2-(allyloxycarbonylamino)-3-methylbutanoate (**I3**)*

[0530] To a stirred solution of the protected acid **I2** (60.6 g, 301.16 mmol, 1.0 eq) and N-hydroxysuccinimide (34.66 g, 301.16 mmol, 1.0 eq) in dry THF (800 mL) was added

dicyclohexylcarbodiimide (62.14 g, 301.16 mmol, 1 eq). The reaction was stirred for 18 hours at room temperature. The reaction mixture was then filtered, the solid washed with THF and the combined filtrate was concentrated under reduced pressure. The residue was re-dissolved in DCM and left to stand at 0°C for 30 minutes. The suspension was filtered and washed with cold DCM. Concentration of the filtrate under reduced pressure afforded the product as a viscous colourless oil (84.7 g, assumed 100% yield) which was used in the next step without further purification. LC/MS (2.194 min (ES^+)), *m/z*: 321.0 $[M+Na]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 8.0 (d, 1H, *J* = 8.3 Hz), 5.97 – 5.87 (m, 1H), 5.30 (ddd, 1H, *J* = 17.2, 3.0, 1.7 Hz), 5.19 (ddd, 1H, *J* = 10.4, 2.7, 1.4 Hz), 4.52 (dt, 2H, *J* = 5.3, 1.4 Hz), 4.32 (dd, 1H, *J* = 8.3, 6.6 Hz), 2.81 (m, 4H), 2.18 (oct, 1H, *J* = 6.7 Hz), 1.00 (d, 6H, *J* = 6.8 Hz),

*(a-iii) (S)-2-((S)-2-(allyloxycarbonylamino)-3-methylbutanamido)propanoic acid (**I4**)*

[0531] A solution of succinimide ester **I3**(12.99 g, 43.55 mmol, 1.0 eq) in THF (50 mL) was added to a solution of L-alanine (4.07 g, 45.73 mmol, 1.05 eq) and $NaHCO_3$ (4.02 g, 47.90 mmol, 1.1 eq) in THF (100 mL) and H_2O (100 mL). The mixture was stirred at room temperature for 72 hours when the THF was removed under reduced pressure. The pH was adjusted to 3-4 with citric acid to precipitate a white gum. After extraction with ethyl acetate (6 x 150 mL), the combined organics were washed with H_2O (200 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Trituration with diethyl ether afforded the product as a white powder which was collected by filtration and washed with diethyl ether (5.78 g, 49%).

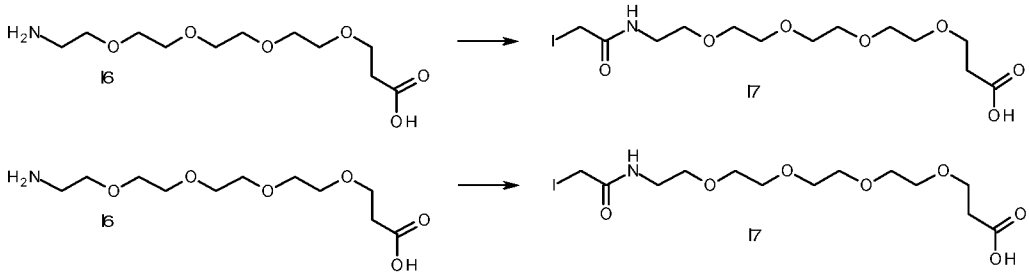
[0532] LC/MS (1.925 min (ES^+)), *m/z*: 273.1 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 12.47 (br s, 1H), 8.17 (d, 1H, *J* = 6.8 Hz), 7.16 (d, 1H, *J* = 9.0 Hz), 5.95 – 5.85 (m, 1H), 5.29 (dd, 1H, *J* = 17.2, 1.7 Hz), 5.17 (dd, 1H, *J* = 10.4, 1.5 Hz), 4.46 (m, 2H), 4.18 (quin, 1H, *J* = 7.2 Hz),

3.87 (dd, 1H, *J* = 9.0, 7.1 Hz), 1.95 (oct, 1H, *J* = 6.8 Hz), 1.26 (d, 3H, *J* = 7.3 Hz), 0.88 (d, 3H, *J* = 6.8 Hz), 0.83 (d, 3H, *J* = 6.8 Hz).

*(a-iv) Allyl (S)-1-((S)-1-(4-(hydroxymethyl)phenylamino)-1-oxopropan-2-ylamino)-3-methyl-1-oxobutan-2-ylcarbamate (**I5**)*

[0533] EEDQ (5.51 g, 22.29 mmol, 1.05 eq) was added to a solution of *p*-aminobenzyl alcohol (2.74 g, 22.29 mmol, 1.05 eq) and acid **I4** (5.78 g, 21.23 mmol, 1 eq) in dry THF (100 mL). and stirred at room temperature for 72 hours. The reaction mixture was then concentrated under reduced pressure and the resulting brown solid was triturated with diethyl ether and filtered with subsequent washing with an excess of diethyl ether to afford the product as an off-white solid (7.1 g, 88 %). LC/MS (1.980 min (ES^+)), *m/z*: 378.0 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 9.89 (br s, 1H), 8.13 (d, 1H, *J* = 7.0 Hz), 7.52 (d, 2H, *J* = 8.5 Hz), 7.26 (m, 1H), 7.23 (d, 2H, *J* = 8.5 Hz), 5.91 (m, 1H), 5.30 (m, 1H), 5.17 (m, 1H), 4.46 (m, 2H), 5.09 (t, 1H, *J* = 5.6 Hz), 4.48 (m, 2H), 4.42 (m, 3H), 3.89 (dd, 1H, *J* = 8.6, 6.8 Hz), 1.97 (m, 1H), 1.30 (d, 3H, *J* = 7.1 Hz), 0.88 (d, 3H, *J* = 6.8 Hz), 0.83 (d, 3H, *J* = 6.7 Hz).

(b)

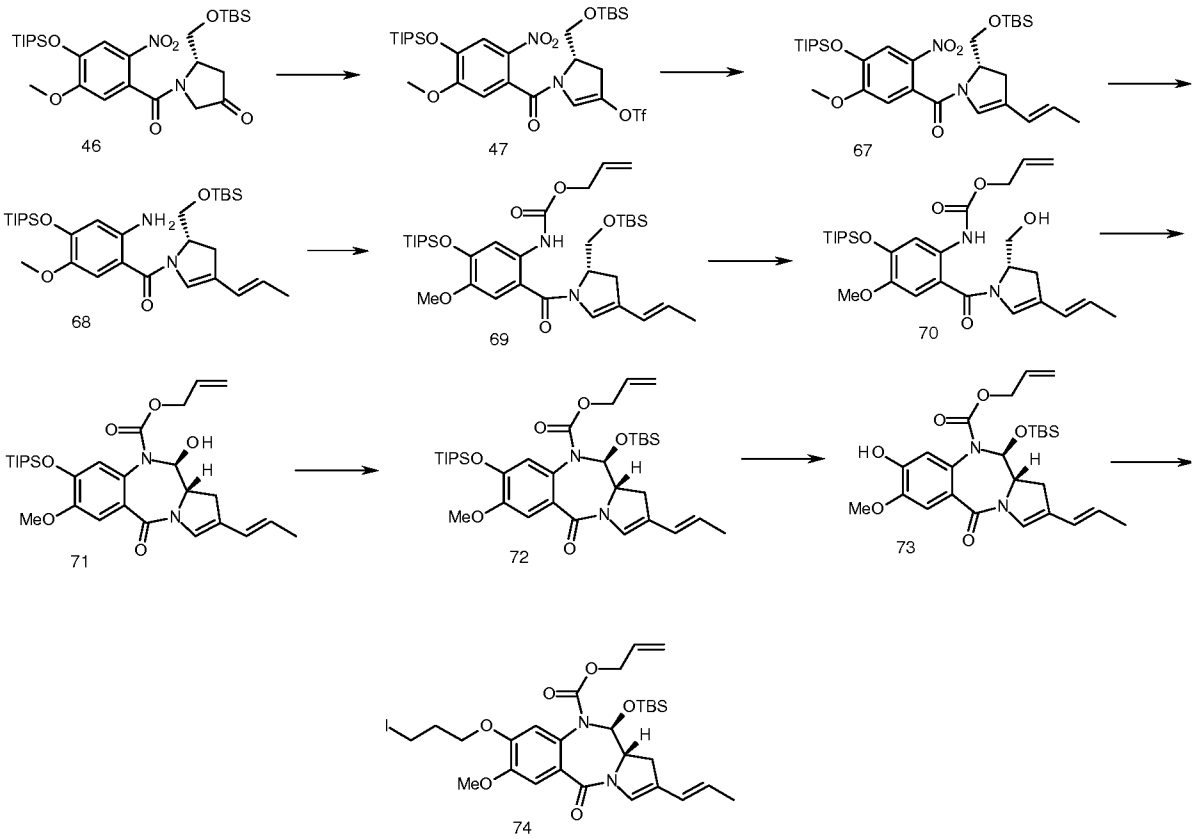


*1-iodo-2-oxo-6,9,12,15-tetraoxa-3-azaoctadecan-18-oic acid (**I7**)*

[0534] A solution of iodoacetic anhydride (0.250 g, 0.706 mmol, 1.1 eq) in dry DCM (1 mL) was added to amino-$PEG_{(4)}$-acid **I6** (0.170 g, 0.642 mmol, 1.0 eq) in DCM (1 mL). The mixture was stirred in the dark at room temperature overnight. The reaction mixture was washed

with 0.1 M HCl, water, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 3% MeOH and 0.1% formic acid in chloroform to 10% MeOH and 0.1% formic acid in chloroform) to afford the product as an orange oil (0.118 g, 42%). LC/MS (1.623 min (ES^+)), *m/z*: 433..98 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.069 (s, 1H), 7.22 (br s, 1H), 3.79 (t, 2H, J = 5.8 Hz), 3.74 (s, 2H), 3.72 – 3.58 (m, 14H), 3.50 – 3.46 (m, 2H), 2.62 (t, 2H, J = 5.8 Hz).

(ii) (11S,11aS)-allyl 11-(tert-butyldimethylsilyloxy)-8-(3-iodopropoxy)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (74)



*(a) (S)-5-((tert-butyldimethylsilyloxy)methyl)-1-(5-methoxy-2-nitro-4-(triisopropylsilyloxy)benzoyl)-4,5-dihydro-1H-pyrrol-3-yl trifluoromethanesulfonate (**47**)*

[0535] Triflic anhydride (28.4 g, 100.0 mmol, 3.0 eq) was added dropwise, over 25 mins, to a vigorously stirred solution of the ketone **46** (19.5 g, 30.0 mmol, 1.0 eq) in DCM (550 mL) containing 2,6-lutidine (14.4 g, 130.0 mmol, 4.0 eq) at -50°C. The reaction mixture was stirred for 1.5 hours when LC/MS indicated complete reaction. The organic phase was washed successively with water (100 mL), saturated sodium bicarbonate (150 mL), brine (50 mL), and the organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 90/10 v/v *n*-hexane/EtOAc) to afford

the product as a pale yellow oil (19.5 g, 82 %). LC/MS (4.391 min (ES^+)), *m/z*: 713.25 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.68 (s, 1H), 6.72 (s, 1H), 6.02 (t, 1H, *J* = 1.9 Hz), 4.75 (m, 1H), 4.05 (m, 2H), 3.87 (s, 3H), 3.15 (ddd, 1H, *J* = 16.2, 10.3, 2.3 Hz), 2.96 (ddd, 1H, *J* = 16.2, 4.0, 1.6 Hz), 1.28 – 1.21 (m, 3H), 1.07 (d, 18H, *J* = 7.2 Hz), 0.88 (s, 9H), 0.09 (s, 3H), 0.08 (s, 3H).

*(b) (S,E)-(2-((tert-butyldimethylsilyloxy)methyl)-4-(prop-1-enyl)-2,3-dihydro-1H-pyrrol-1-yl)(5-methoxy-2-nitro-4-(triisopropylsilyloxy)phenyl)methanone (**67**)*

[0536] Tetrakis(triphenylphosphine)palladium(0) (0.41 g, 0.35 mmol, 0.03 eq) was added to a mixture of the triflate **47** (8.4 g, 11.8 mmol, 1.0 eq), *E*-1-propene-1-ylboronic acid (1.42 g, 16.5 mmol, 1.4 eq) and potassium phosphate (5.0 g, 23.6 mmol, 2.0 eq) in dry dioxane (60 mL) under a nitrogen atmosphere. The mixture was stirred at 25°C for 120 mins when LC/MS indicated complete reaction. Ethyl acetate (120 mL) and water (120 mL) were added, the organic phase was removed, washed with brine (20 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 95/5 v/v *n*-hexane/EtOAc to 90/10 v/v *n*-hexane/EtOAc) to afford the product as a yellow foam (4.96 g, 70 %). LC/MS (4.477 min (ES^+)), *m/z*: 605.0 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.67 (s, 1H), 6.74 (s, 1H), 5.93 (d, 1H, *J* = 15.4 Hz), 5.67 (s, 1H), 4.65 (m, 1H), 4.04 (m, 2H), 3.86 (s, 3H), 2.85 (m, 1H), 2.71 (m, 1H), 1.72 (dd, 3H, *J* = 6.8, 1.0 Hz), 1.30 – 1.22 (m, 3H), 1.07 (d, 18H, *J* = 7.2 Hz), 0.87 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H).

*(c) (S,E)-(2-amino-5-methoxy-4-(triisopropylsilyloxy)phenyl)(2-((tert-butyldimethylsilyloxy)methyl)-4-(prop-1-enyl)-2,3-dihydro-1H-pyrrol-1-yl)methanone (**68**)*

[0537] Zinc dust (22.0 g, 0.33 mol, 37 eq) was added, in portions over 20 mins, to a solution of the propenyl intermediate **67** (5.5 g, 9.1 mmol, 1.0 eq) in 5% v/v formic acid / ethanol (55 mL), using an ice bath to maintain the temperature between 25-30°C. After 30 mins, the

reaction mixture was filtered through a short bed of celite®. The celite® was washed with ethyl acetate (65 mL) and the combined organics were washed successively with water (35 mL), saturated sodium bicarbonate (35 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 90/10 v/v *n*-hexane/EtOAc) to afford the product as a pale yellow oil (3.6 g, 69.0 %). LC/MS (4.439 min (ES^+)), *m/z*: 575.2 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 6.75 (m, 1H), 6.40 (br s, 1H), 6.28 (m, 1H), 6.11 (d, 1H, *J* = 15.4 Hz), 5.53 (m, 1H), 4.67 (m, 1H), 4.36 (m, 2H), 3.93 (br s, 1H), 3.84 (br s, 1H), 3.73 (s, 3H), 2.86 (dd, 1H, *J* = 15.7, 10.4 Hz), 2.73 (dd, 1H, *J* = 15.9, 4.5 Hz), 1.80 (dd, 3H, *J* = 6.8, 1.3 Hz), 1.35 – 1.23 (m, 3H), 1.12 (d, 18H, *J* = 7.3 Hz), 0.89 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H).

*(d) (S,E)-allyl 2-(2-((tert-butyldimethylsilyloxy)methyl)-4-(prop-1-enyl)-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-(triisopropylsilyloxy)phenylcarbamate (**69**)*

[0538] Allyl chloroformate (0.83 g, 6.88 mmol, 1.1 eq) was added to a solution of the amine **68** (3.6 g, 6.26 mmol, 1.0 eq) in dry DCM (80 mL) containing dry pyridine (1.09 g, 13.77 mmol, 2.2 eq) at -78°C. The dry ice was removed and the reaction mixture allowed to warm to room temperature. After stirring for a further 15 minutes, LC/MS indicated complete reaction. The organic phase was washed successively with 0.01N HCl (50 mL), saturated sodium bicarbonate (50 mL), brine (10 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to leave a pale yellow oil which was used in the next step without further purification (4.12g, assumed 100% yield). LC/MS (4.862 min (ES^+)), *m/z*: 659.2 $[M+H]^+$.

*(e)(S,E)-allyl 2-(2-(hydroxymethyl)-4-(prop-1-enyl)-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-(triisopropylsilyloxy)phenylcarbamate (**70**)*

[0539] The crude intermediate **69** (assumed 100% yield, 4.12 g, 6.25 mmol, 1.0 eq) was dissolved in a mixture of acetic acid (70 mL), methanol (10 mL), THF (10 mL) and water (20

mL) and allowed to stir at room temperature. After 6 hours the reaction mixture was diluted with ethyl acetate (500 mL) and washed successively with water (2 x 500 mL), saturated sodium bicarbonate (300 mL) and brine (50 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 1/99 v/v methanol/DCM to 5/95 v/v methanol/DCM) to afford the product as a yellow oil and a further 1 g of unreacted starting material was recovered. This material was subjected to the same reaction conditions as above, but was left stirring for 16 h. After work up and purification, additional product was isolated (2.7 g, 79%, 2 steps) LC/MS (3.742 min (ES^+)), *m/z*: 545.2 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.38 (m, 1H), 7.72 (m, 1H), 6.81 (s, 1H), 6.37 (m, 1H), 6.10 (d, 1H, *J* = 15.8 Hz), 5.97 (m, 1H), 5.53 (m, 1H), 5.36 (ddd, 1H, *J* = 17.2, 3.1, 1.5 Hz), 5.25 (ddd, 1H, *J* = 10.4, 2.5, 1.3 Hz), 4.78 (m, 1H), 4.65 (dt, 2H, *J* = 5.7, 1.3 Hz), 3.84 (m, 3H), 3.79 (s, 3H), 3.04 (dd, 1H, *J* = 16.7, 10.5 Hz), 2.40 (dd, 1H, *J* = 16.0, 4.5 Hz), 1.82 (dd, 3H, *J* = 6.8, 1.0 Hz), 1.36 – 1.26 (m, 3H), 1.14 (d, 18H, *J* = 7.3 Hz).

*(f) (11S,11aS)-allyl 11-hydroxy-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-8-(triisopropylsilyloxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**71**)*

[0540] Dry dimethyl sulfoxide (1.16 g, 14.87 mmol, 3.0 eq) was added dropwise to a solution of oxalyl chloride (0.94 g, 7.43 mmol, 1.5 eq) in DCM (25 mL) at -78°C under an atmosphere of nitrogen. Maintaining the temperature at -78°C, after 10 mins a solution of the primary alcohol **70** (2.7 g, 4.96 mmol, 1.0 eq) in DCM (20 mL) was added dropwise. After a further 15 mins, dry triethylamine (2.5g, 24.78 mmol, 5.0 eq) was added, and the reaction mixture allowed to warm to room temperature. The reaction mixture was washed successively with cold 0.1N HCl (50 mL), saturated sodium hydrogen carbonate (50 mL) and brine (10 mL) and the organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure to

afford the product as a yellow oil which was used in the next step without further purification (2.68g, assumed 100% yield). LC/MS (3.548 min (ES^+)), *m/z*: 543.2 $[M+H]^+$.

*(g) (11S,11aS)-allyl 11-(tert-butyldimethylsilyloxy)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-8-(triisopropylsilyloxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**72**)*

[0541] *Tert*-butyldimethylsilyltrifluoromethane sulfonate (3.93 g, 14.87 mmol, 3.0 eq) was added to a solution of the carbinolamine **71** (assumed 100% yield, 2.68 g, 4.96 mmol, 1.0 eq) and 2,6-lutidine (2.12 g, 19.83 mmol, 4.0 eq) in dry DCM (40 mL) at 0°C under an atmosphere of nitrogen. After 10 minutes, the reaction mixture was allowed to warm to room temperature and stirred for a further 60 minutes. The organic phase was washed successively with water (10 mL), saturated sodium bicarbonate (10 mL) and brine (5 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, chloroform to 2/98 v/v Methanol/chloroform) to afford the product as a yellow oil (2.0g, 63%, 2 steps). LC/MS (4.748 min (ES^+)), *m/z*: 657.2 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.19 (s, 1H), 6.86 (m, 1H), 6.66 (s, 1H), 6.22 (d, 1H, *J* = 15.4 Hz), 5.81 (d, 1H, *J* = 8.8 Hz), 5.78 (m, 1H), 5.48 (m, 1H), 5.11 (d, 1H, *J* = 5.0 Hz), 5.08 (m, 1H), 4.58 (dd, 1H, *J* = 13.4, 5.4 Hz), 4.35 (dd, 1H, *J* = 13.2, 5.7 Hz), 3.83 (s, 3H), 3.76 (s, 1H), 3.00 (dd, 1H, *J* = 15.6, 11.0 Hz), 2.53 (m, 1H), 1.81 (dd, 3H, *J* = 6.8, 0.9 Hz), 1.30 – 1.18 (m, 3H), 1.08 (d, 9H, *J* = 2.3 Hz), 1.06 (d, 9H, *J* = 2.3 Hz), 0.86 (s, 9H), 0.25 (s, 3H), 0.18 (s, 3H).

*(h) (11S,11aS)-allyl 11-(tert-butyldimethylsilyloxy)-8-hydroxy-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**73**)*

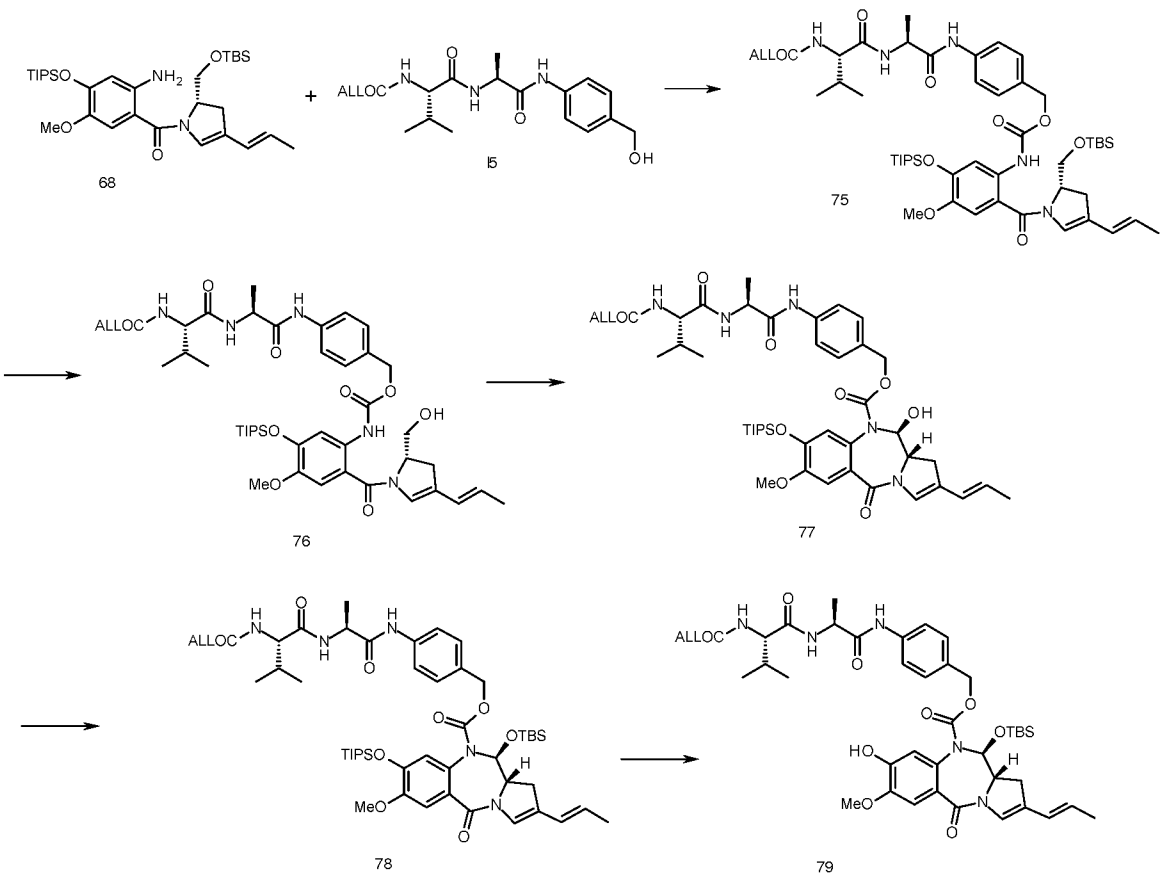
[0542] Lithium acetate dihydrate (0.31 g, 3.04 mmol, 1.0 eq) was added to a solution of the diazepine **72** (2.0 g, 3.04 mmol, 1.0 eq) in wet DMF (20 mL) at 25°C and stirred for 4 hours.

The reaction mixture was diluted with ethyl acetate (200 mL) and washed successively with 0.1M citric acid (50 mL, pH 3), water (50 mL) and brine (10 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 50/50 v/v *n*-hexane/EtOAc to 25/75 v/v *n*-hexane/EtOAc) to afford the product as a pale yellow solid (0.68g, 45 %). LC/MS (3.352 min (ES^+)), *m/z*: 501.1 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.02 (s, 1H), 6.66 (m, 1H), 6.53 (s, 1H), 6.03 (d, 1H, *J* = 15.5 Hz), 5.80 (s, 1H), 5.63 (d, 1H, *J* = 8.9 Hz), 5.55 (m, 1H), 5.29 (m, 1H), 4.87 (m, 2H), 4.39 (dd, 1H, *J* = 13.5, 4.2 Hz), 4.20 (dd, 1H, *J* = 13.2, 5.7 Hz), 3.73 (s, 3H), 3.59 (m, 1H), 2.81 (dd, 1H, *J* = 16.1, 10.5 Hz), 2.35 (d, 1H, *J* = 15.7 Hz), 1.61 (d, 3H, *J* = 6.4 Hz), 0.67 (s, 9H), 0.05 (s, 3H), 0.00 (s, 3H).

*(i) (11S,11aS)-allyl 11-(tert-butyldimethylsilyloxy)-8-(3-iodopropoxy)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**74**)*

[0543] Diiodopropane (0.295 g, 1.00 mmol, 5.0 eq) and potassium carbonate (0.028 g, 0.20 mmol, 1.0 eq) were added to a solution of the phenol **33** (0.100 g, 0.020 mmol, 1.0 eq) in dry acetone (5 mL). The reaction mixture was heated at 60°C for 6 hours when LC/MS showed complete reaction. The reaction mixture was concentrated to dryness under reduced pressure and the residue was purified by flash chromatography (silica gel, 75/25 v/v *n*-hexane/EtOAc to 50/50 v/v *n*-hexane/EtOAc) to afford the product as a colourless oil (0.074 g, 56%). LC/MS (3.853 min (ES^+)), *m/z*: 669.0 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.26 (s, 1H), 6.90 (s, 1H), 6.68 (s, 1H), 6.24 (d, 1H, *J* = 15.3 Hz), 5.87 (d, 1H, *J* = 8.9 Hz), 5.78 (m, 1H), 5.53 (m, 1H), 5.12 (m, 2H), 4.65 (m, 2H), 4.41 (m, 1H), 4.11 (m, 1H), 3.93 (s, 3H), 3.81 (m, 1H), 3.40 (t, 2H, *J* = 6.7 Hz), 3.05 (dd, 1H, *J* = 16.3, 10.1 Hz), 2.57 (m, 1H), 2.34 (m, 2H), 1.84 (d, 3H, *J* = 6.6 Hz), 0.92 (s, 9H), 0.28 (s, 3H), 0.26 (s, 3H).

(iii) (11S,11aS)-4-((S)-2-((S)-2-(allyloxycarbonylamino)-3-methylbutanamido)propanamido)benzyl 11-(tert-butyldimethylsilyloxy)-8-hydroxy-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate 79)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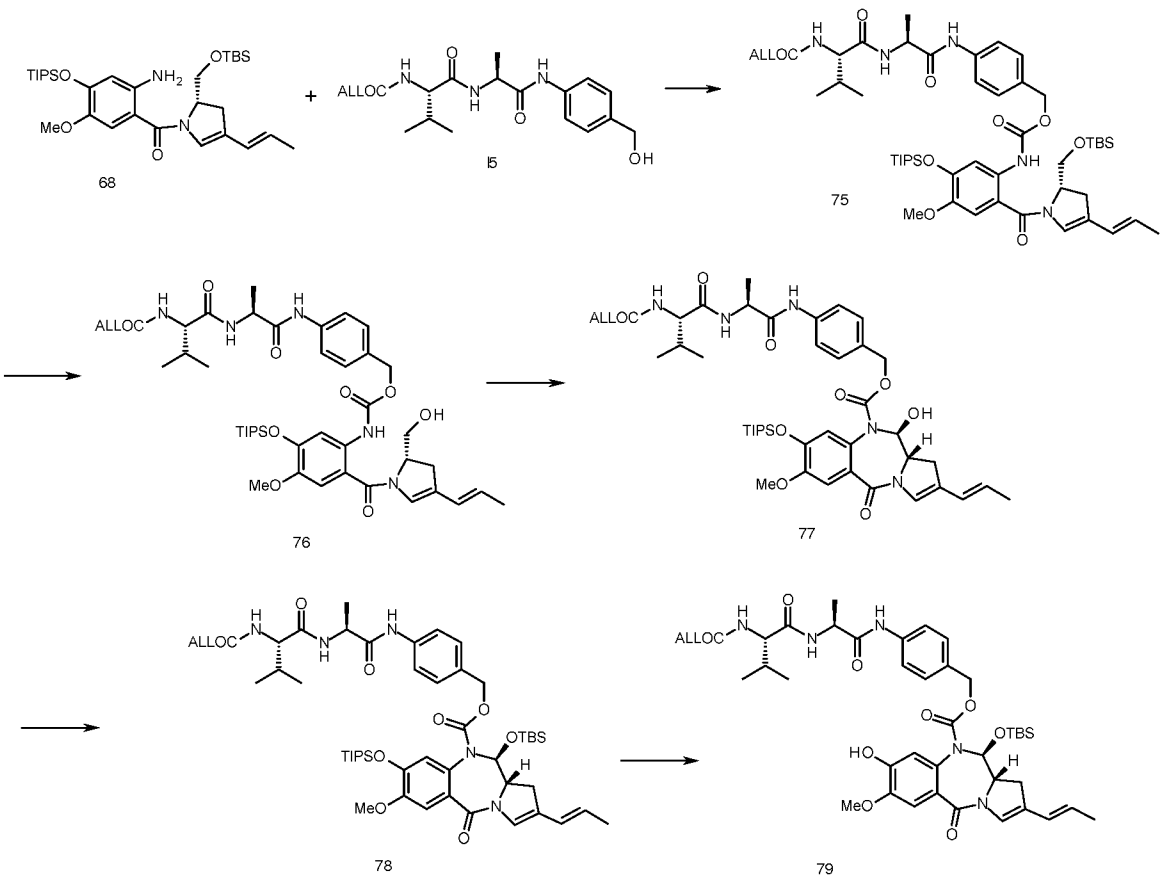


*(a) Allyl ((S)-1-(((S)-1-((4-((((2-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-((E)-prop-1-en-1-yl)-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**75**)*

[0544] Triethylamine (0.256 mL, 1.84 mmol, 2.2 eq) was added to a stirred solution of the amine **68** (0.480 g, 0.835 mmol, 1.0 eq) and triphosgene (0.089 g, 0.301 mmol, 0.36 eq) in dry THF (15 mL) at 5°C (ice bath). The progress of the isocyanate reaction was monitored by periodically removing aliquots from the reaction mixture and quenching with methanol and performing LCMS analysis. Once the isocyanate reaction was complete a solution of Alloc-Val-Ala-PABOH **I5** (0.473 g, 1.25 mmol, 1.5 eq) and triethylamine (0.174 mL, 1.25 mmol, 1.5 eq) in dry THF (10 mL) was rapidly added by injection to the freshly prepared isocyanate. The reaction

was allowed to stir at 40°C for 4 hours followed by stirring at room temperature overnight. The mixture was concentrated under reduced pressure, and purified by flash chromatography (silica gel, 20/80 v/v *n*-hexane/EtOAc to 50/50 v/v *n*-hexane/EtOAc, then 1/99 v/v DCM/MeOH to 5/95 v/v DCM/MeOH) to afford the product as a yellow solid (0.579 g, 71%). LC/MS (4.468 min (ES^+)), *m/z*: 978.55 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.63 (br s, 1H), 8.42 (s, 1H), 7.78 (br s, 1H), 7.53 (d, 2H, *J* = 8.1 Hz), 7.31 (d, 2H, *J* = 8.6 Hz), 6.76 (s, 1H), 6.59 (d, 1H, *J* = 7.6 Hz), 6.36 (br s, 1H), 6.04 (d, 1H, *J* = 15.9 Hz), 5.90 (m, 1H), 5.55 (m, 1H), 5.33 – 5.21 (m, 3H), 5.10 (s, 2H), 4.66 (m, 2H), 4.57 (dd, 2H, *J* = 5.6, 1.0 Hz), 3.98 (dd, 1H, *J* = 7.3, 6.8 Hz), 3.90 (m, 1H), 3.81 (m, 1H), 3.78 (s, 3H), 2.82 (dd, 1H, *J* = 15.4, 9.6 Hz), 2.72 (dd, 1H, *J* = 15.9, 3.5 Hz), 2.17 (m, 1H), 1.78 (dd, 3H, *J* = 6.5, 0.8 Hz), 1.46 (d, 3H, *J* = 7.1 Hz), 1.29 (m, 3H), 1.11 (d, 18H, *J* = 7.1 Hz), 0.97 (d, 3H, *J* = 6.8 Hz), 0.92 (d, 3H, *J* = 6.8 Hz), 0.83 (s, 9H), 0.04 (s, 3H), 0.01 (s, 3H).

*(b) Allyl ((S)-1-(((S)-1-((4-((((2-((S)-2-(hydroxymethyl)-4-((E)-prop-1-en-1-yl)-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5-((triisopropylsilyl)oxy)phenyl)carbamoyl)oxy)methyl)phenyl)amino)-1-oxopropan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (**76**)*

[0545] The silyl ether **75** (1.49 g, 1.52 mmol, 1.0 eq) was dissolved in a 7:1:1:2 mixture of acetic acid/ methanol/ tetrahydrofuran/ water (14:2:2:4 mL) and allowed to stir at room temperature. After 2 hours the reaction was diluted with EtOAc (100 mL), washed sequentially with water, aq. sodium bicarbonate then brine. The organic phase was then dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 100/0 then 99/1 to 92/8 v/v DCM/ MeOH) to afford the product as an orange solid (1.2 g, 92%). LC/MS (3.649 min (ES^+)), *m/z*: 865.44 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.44 (s, 1H), 8.35 (s, 1H), 7.69 (br s, 1H), 7.53 (d, 2H, *J* = 8.7 Hz), 7.32 (d, 2H,

J = 8.3 Hz), 6.78 (s, 1H), 6.56 (m, 2H), 6.32 (br s, 1H), 6.05 (d, 1H, *J* = 14.9 Hz), 5.90 (m, 1H), 5.56 (m, 1H), 5.30 (m, 2H), 5.22 (m, 1H), 5.10 (d, 2H, *J* = 3.1 Hz), 4.73 (m, 1H), 4.64 (m, 1H), 4.57 (d, 2H, *J* = 5.8 Hz), 4.01 (m, 1H), 3.79 (m, 2H), 3.76 (s, 3H), 2.98 (dd, 1H, *J* = 16.3, 10.2 Hz), 2.38 (dd, 1H, *J* = 16.6, 4.1 Hz), 2.16 (m, 1H), 1.78 (dd, 3H, *J* = 6.8, 0.9 Hz), 1.46 (d, 3H, *J* = 7.1 Hz), 1.29 (m, 3H), 1.11 (d, 18H, *J* = 7.4 Hz), 0.97 (d, 3H, *J* = 6.7 Hz), 0.92 (d, 3H, *J* = 6.8 Hz).

(c) (11S,11aS)-4-((S)-2-((S)-2-(allyloxycarbonylamino)-3-methylbutanamido)propanamido)benzyl 11-hydroxy-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-8-(triisopropylsilyloxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (77)

[0546] Dry dimethyl sulfoxide (0.180 g, 2.3 mmol, 3.0 eq) was added dropwise to a solution of oxalyl chloride (0.147 g, 1.1mmol, 1.5 eq) in DCM (10 mL) at -78°C under an atmosphere of nitrogen. Maintaining the temperature at -78°C, after 20 minutes, a solution of the primary alcohol **76** (0.666 g, 0.77 mmol, 1.0 eq) in DCM (10 mL) was added dropwise. After a further 15 minutes, dry triethylamine (0.390 g, 3.85 mmol, 5.0 eq) was added, and the reaction mixture allowed to warm to room temperature. The reaction mixture was washed successively with cold 0.1N HCl (10 mL), saturated sodium hydrogen carbonate (10 mL) and brine (5 mL). The organic layer was then dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was then purified by flash chromatography (silica gel, 50/50 v/v *n*-hexane/EtOAc to 25/75 v/v *n*-hexane/EtOAc) to afford the product as a white solid (0.356g, 54 %). LC/MS (3.487 min (ES^+)), *m/z*: 862.2 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.34 (br s, 1H), 7.47 (d, 2H, *J* = 7.6 Hz), 7.17 (s, 1H), 7.14 (d, 2H, *J* = 7.5 Hz), 6.86 (br s, 1H), 6.65 (br s, 1H), 6.42 (d, 1H, *J* = 7.6 Hz), 6.22 (d, 1H, *J* = 14.4 Hz), 5.80 (m, 1H), 5.40 (m, 1H), 5.53 (m, 1H), 5.32 (m, 1H), 5.21 (d, 2H, *J* = 9.6 Hz), 5.06 (d, 1H, *J* = 12.3 Hz), 4.90 (m, 1H), 4.58 (m, 3H), 3.98 (m, 1H), 3.84 (m,

1H), 3.81 (s, 3H), 3.50 (m, 1H), 3.05 (dd, 1H, *J* = 16.0, 10.3 Hz), 2.76 (m, 1H), 2.15 (m, 1H), 1.80 (dd, 3H, *J* = 6.7, 0.8 Hz), 1.44 (d, 3H, *J* = 7.1 Hz), 1.16 (m, 3H), 1.01 (d, 18H, *J* = 6.6 Hz), 0.96 (d, 3H, *J* = 6.8 Hz), 0.92 (d, 3H, *J* = 6.8 Hz).

*(d) (11S,11aS)-4-((S)-2-((S)-2-(allyloxycarbonylamino)-3-methylbutanamido)propanamido)benzyl 11-(tert-butyldimethylsilyloxy)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-8-(triisopropylsilyloxy)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**78**)*

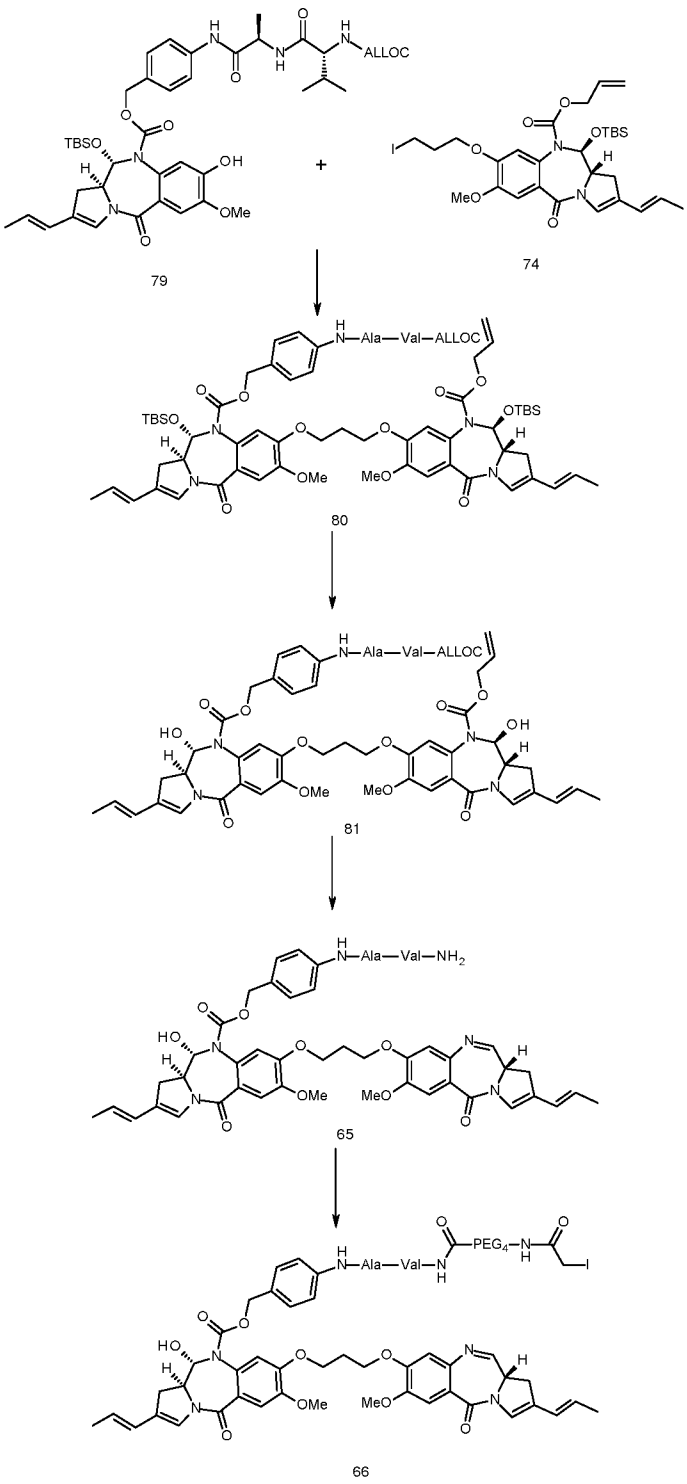
[0547] *Tert*-butyldimethylsilyltrifluoromethane sulfonate (0.46 g, 1.74mmol, 3.0 eq) was added to a solution of secondary alcohol **77** (0.5 g, 0.58 mmol, 1.0 eq) and 2,6-lutidine (0.25 g, 2.32 mmol, 4.0 eq) in dry DCM (10 mL) at 0°C under an atmosphere of nitrogen. After 10 minutes, the reaction mixture was allowed to warm to room temperature and stirred for a further 120 mins. The organic phase was then washed successively with water (10 mL), saturated sodium bicarbonate (10 mL) and brine (5 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 50/50 v/v *n*-hexane/EtOAc) to afford the product as a white solid (0.320 g, 57 %). LC/MS (4.415 min (ES^+)), *m/z*: 976.52 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.31 (br s, 1H), 7.48 (d, 2H, *J* = 8.0 Hz), 7.21 (s, 1H), 7.14 (d, 2H, *J* = 8.3 Hz), 6.89 (s, 1H), 6.65 (s, 1H), 6.38 (d, 1H, *J* = 7.3 Hz), 6.25 (d, 1H, *J* = 14.6 Hz), 5.93 (m, 1H), 5.85 (d, 1H, *J* = 8.8 Hz), 5.50 (m, 1H), 5.34 (m, 1H), 5.24 (m, 2H), 5.15 (d, 1H, *J* = 12.5 Hz), 4.86 (d, 1H, *J* = 12.2 Hz), 4.62 (m, 3H), 4.01 (m, 1H), 3.86 (s, 3H), 3.78 (m, 1H), 3.04 (m, 1H), 2.56 (m, 1H), 2.20 (m, 1H), 1.84 (dd, 3H, *J* = 6.6, 0.7 Hz), 1.48 (d, 3H, *J* = 6.8 Hz), 1.20 (m, 3H), 1.05 (d, 9H, *J* = 2.9 Hz), 1.03 (d, 9H, *J* = 2.9 Hz), 0.99 (d, 3H, *J* = 6.8 Hz), 0.95 (d, 3H, *J* = 6.8 Hz), 0.88 (s, 9H), 0.27 (s, 3H), 0.14 (s, 3H).

(e) (11S,11aS)-4-((S)-2-((S)-2-(allyloxycarbonylamino)-3-methylbutanamido)propanamido)benzyl 11-(tert-butyldimethylsilyloxy)-8-hydroxy-7-

*methoxy-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**79**)*

[0548] Lithium acetate dihydrate (0.010 g, 0.10 mmol, 1.0 eq) was added to a solution of the silyl ether **78** (0.100 g, 0.10 mmol, 1.0 eq) in wet DMF (2 mL) at 25°C for 3 hours. The reaction mixture was then diluted with ethyl acetate (20 mL) and washed successively with 0.1M citric acid (20 mL, pH 3), water (20 mL) and brine (5 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 5/95 v/v methanol/DCM) to afford the product as a pale yellow oil (0.070 g, 83 %). LC/MS (3.362 min (ES^+)), *m/z*: 820.2 $[M+H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.39 (s, 1H), 7.48 (d, 2H, *J* = 8.2 Hz), 7.25 (s, 1H), 7.12 (d, 2H, *J* = 8.1 Hz), 6.88 (s, 1H), 6.68 (s, 1H), 6.47 (d, 1H, *J* = 7.6 Hz), 6.24 (d, 1H, *J* = 15.2 Hz), 6.03 (s, 1H), 5.92 (m, 1H), 5.84 (d, 1H, *J* = 8.9 Hz), 5.50 (m, 1H), 5.34 (m, 1H), 5.26 (m, 2H), 5.18 (d, 1H, *J* = 12.3 Hz), 4.80 (d, 1H, *J* = 12.4 Hz), 4.66 – 4.60 (m, 3H), 4.02 (m, 1H), 3.95 (s, 3H), 3.81 (m, 1H), 3.03 (m, 1H), 2.57 (m, 1H), 2.19 (m, 1H), 1.84 (dd, 3H, *J* = 6.8, 0.8 Hz), 1.48 (d, 3H, *J* = 7.1 Hz), 1.00 (d, 3H, *J* = 6.8 Hz), 0.95 (d, 3H, *J* = 6.8 Hz), 0.87 (s, 9H), 0.26 (s, 3H), 0.12 (s, 3H).

(iv) (11S,11aS)-4-((20S,23S)-1-iodo-20-isopropyl-23-methyl-2,18,21-trioxo-6,9,12,15-tetraoxa-3,19,22-triazatetracosanamido)benzyl 11-hydroxy-7-methoxy-8-(3-((S)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yloxy)propoxy)-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (66, D)



(a) (11S,11aS)-allyl 8-(3-((11S,11aS)-10-((4-((R)-2-((R)-2-(allyloxycarbonylamino)-3-methylbutanamido)propanamido)benzyloxy)carbonyl)-11-(tert-butyldimethylsilyloxy)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-

*a][1,4]diazepin-8-yloxy)propoxy)-11-(tert-butyldimethylsilyloxy)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**80**)*

[0549] Potassium carbonate (0.030 g, 0.21 mmol, 1.0 eq) was added to a solution of the phenol **79** (0.175 g, 0.21 mmol, 1.0 eq) and the iodo linker **74** (0.214 g, 0.32 mmol, 1.5 eq) in acetone (10 mL). The reaction mixture was heated under a nitrogen atmosphere at 75°C in a sealed flask for 17 hours. The reaction mixture was concentrated to dryness under reduced pressure and purified by flash chromatography (silica gel, 2/98 v/v methanol/DCM to 5/95 v/v methanol/DCM) to afford the product as a pale yellow solid (0.100 g, 35%). LC/MS (4.293 min (ES^+)), *m/z*: 1359.13 $[M]^+$.

*(b) (11S,11aS)-allyl 8-(3-((11S,11aS)-10-((4-((R)-2-((R)-2-(allyloxycarbonylamino)-3-methylbutanamido)propanamido)benzyloxy)carbonyl)-11-hydroxy-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-5,10,11,11a-tetrahydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yloxy)propoxy)-11-hydroxy-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**81**)*

[0550] Tetra-n-butylammonium fluoride (1M, 0.22 mL, 0.22 mmol, 2.0 eq) was added to a solution of silyl ether **80** (0.150 g, 0.11 mmol, 1.0 eq) in dry THF (2 mL). The reaction mixture was stirred at room temperature for 20 minutes, after which LC/MS indicated complete reaction. The reaction mixture was diluted with ethyl acetate (10 mL) and washed sequentially with water (5 mL) and brine (5 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure to leave a yellow solid. Purification by flash chromatography (silica gel, 6/94 v/v methanol/DCM to 10/90 v/v methanol/DCM) afforded the product as a pale yellow solid (0.090 g, 73%). LC/MS (2.947 min (ES^+)), *m/z*: 1154.0 $[M+Na]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.39 (br s, 1H), 7.39 (d, 2H, *J* = 7.6 Hz), 7.18 (d, 2H, *J* = 10.6 Hz), 7.10 (m, 3H), 6.86 (d, 2H, *J* = 10.0 Hz), 6.74 (s, 1H), 6.55 (s, 1H), 6.22 (dd, 2H, *J* = 15.3, 6.6 Hz), 5.85 (m, 2H),

5.74 (m, 3H), 5.52 (m, 2H), 5.22 (m, 1H), 5.00 (m, 2H), 4.57 (m, 6H), 4.41 (m, 2H), 4.09 (m, 4H), 3.85 (m, 11H), 3.06 (m, 2H), 2.76 (m, 2H), 2.20 (m, 2H), 2.08 (m, 1H), 1.79 (d, 6H, J = 6.4 Hz), 1.40 (d, 3H, J = 6.1 Hz), 0.90 (m, 6H).

*(c) (11S,11aS)-4-((R)-2-((R)-2-amino-3-methylbutanamido)propanamido)benzyl 11-hydroxy-7-methoxy-8-(3-((S)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yloxy)propoxy)-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**65**)*

[0551] Tetrakis(triphenylphospene)palladium(0) (0.005 g, 0.005 mmol, 0.06 eq) was added to a solution of the bis-carbinolamine **81** (0.090 g, 0.08 mmol, 1.0 eq) and pyrrolidine (16 μL, 0.20 mmol, 2.5 eq) in dry DCM (5 mL). After 20 minutes, the reaction mixture was diluted with DCM (10 mL) and washed sequentially with saturated ammonium chloride (5 mL) and brine (5 mL), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to leave the crude product as a pale yellow solid which was used in the next step without further purification (0.075 g, assumed 100% yield). LC/MS (2.060 min (ES^+)), *m/z*: 947.2 $[M+H]^+$.

*(d) (11S,11aS)-4-((20S,23S)-1-iodo-20-isopropyl-23-methyl-2,18,21-trioxo-6,9,12,15-tetraoxa-3,19,22-triazatetracosanamido)benzyl 11-hydroxy-7-methoxy-8-(3-((S)-7-methoxy-5-oxo-2-((E)-prop-1-enyl)-5,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-8-yloxy)propoxy)-5-oxo-2-((E)-prop-1-enyl)-11,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-10(5H)-carboxylate (**66, D**)*

[0552] EDCI (0.015 g, 0.08 mmol, 1.0 eq) was added to a solution of amine **65** (assumed 100% yield 0.075 g, 0.08 mmol, 1.0 eq) and iodoacetamide-PEG_4-acid **I7** (0.034 g, 0.08 mmol, 1.0 eq) in dry dichloromethane (5 mL) and the reaction was stirred in the dark. After 50 minutes, a further amount of iodoacetamide-PEG_4-acid **I7** (0.007 g, 0.016 mmol, 0.2 eq) was added along with a further amount of EDCI (0.003 g, 0.016 mmol, 0.2 eq). After a total of 2.5 hours, the reaction mixture was diluted with dichloromethane (15 mL) and washed sequentially with water (10 mL) and brine (10 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting residue was purified by flash chromatography (silica gel, Chloroform 100% to 90:10 v/v Chloroform:Methanol). Pure fractions were combined to afford

the product (0.0254 g, 23%, 2 steps). The crude fractions were collected and purified by preparative TLC (silica gel, 90:10 v/v Chloroform:Methanol) to afford a second batch of product (0.0036 g, 3%, 2 steps). LC/MS (2.689 min (ES^+)), *m/z*: 681.0 1/2[M+2H]$^+$.

Example 20

General Experimental Methods

[0553] Reaction progress was monitored by thin-layer chromatography (TLC) using Merck Kieselgel 60 F254 silica gel, with fluorescent indicator on aluminium plates. Visualisation of TLC was achieved with UV light or iodine vapour unless otherwise stated. Flash chromatography was performed using Merck Kieselgel 60 F254 silica gel. Extraction and chromatography solvents were bought and used without further purification from Fisher Scientific, U.K. All chemicals were purchased from Aldrich, Lancaster or BDH.

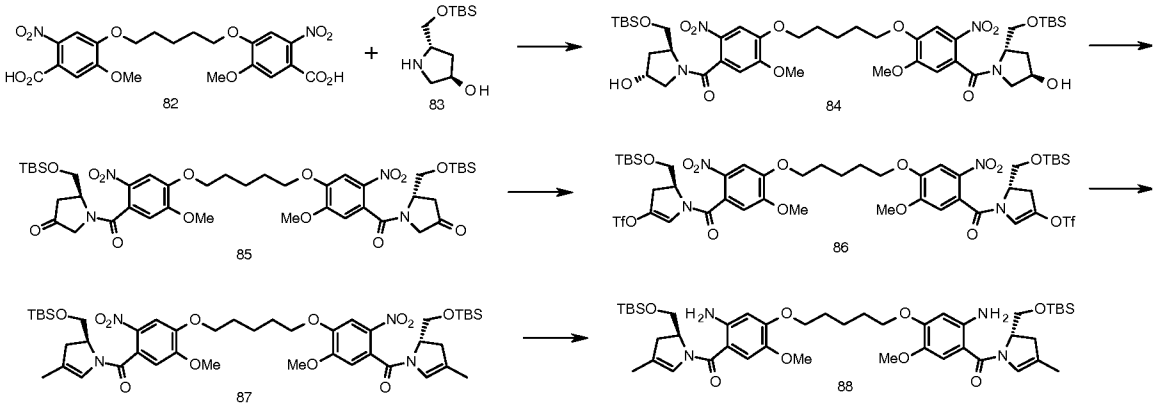
[0554] Proton NMR chemical shift values were measured on the delta scale at 400 MHz using a Bruker AV400. The following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; br, broad. Coupling constants are reported in Hz. Unless otherwise stated, column chromatography (by the flash procedure) were performed on Merck Kieselgel silica (Art. 9385). Mass spectroscopy (MS) data were collected using a Waters Micromass LCT instrument coupled to a Waters 2795 HPLC separations module. Thin Layer Chromatography (TLC) was performed on silica gel aluminium plates (Merck 60, F_{254}). All other chemicals and solvents were used as supplied without further purification.

[0555] LCMS data were obtained using a Shimadzu Nexera series LC/MS with a Shimadzu LCMS-2020 quadrupole MS, with Electrospray ionisation. Mobile phase A - 0.1% formic acid in water. Mobile phase B - 0.1% formic acid in acetonitrile. Flow rate of 0.80ml/min. Gradient from 5% B rising up to 100% B over 2.00 min, remaining at 100% B for 0.50 min and then back down to 5% B over 0.05 min (held for 0.45 min). The total run time is 3 min. Column: Waters Aquity UPLC BEH Shield RP18 1.7 µm, 2.1 x 50mm; (System 1).

[0556] Or, gradient from 5% B rising up to 100% B over 10.00 min, remaining at 100% B for 2.00 min and then back down to 5% B over 0.10 minutes (held for 2.90 min). The total run time is 15 minutes. Column: Gemini-NX 3u C18 110A, 100 x 2.00mm; (System 2).

[0557] Chromatograms based on UV detection at 254nm. Mass Spectra were achieved using the MS in positive mode.

(i) (S)-((pentane-1,5-diylbis(oxy))bis(2-amino-5-methoxy-4,1-phenylene))bis(((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone) (88)



*(a) (S,R)-((pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitro-4,1-phenylene))bis(((2S,4R)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-hydroxypyrrolidin-1-yl)methanone) (**84**)*

[0559] Anhydrous DMF (approx. 0.5 mL) was added dropwise to a stirred suspension of 4,4'-(pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitrobenzoic acid) (**82**) (36.64 g, 74.0 mmol) and oxalyl chloride (18.79 mL, 0.222 mol, 3.0 eq.) in anhydrous DCM (450 mL) until vigorous effervescence occurred and the reaction mixture was left to stir overnight. The reaction mixture was evaporated to dryness, and triturated with diethyl ether. The resulting yellow precipitate was filtered from solution, washed with diethyl ether (100 mL) and immediately added to a solution of (3R,5S)-5-((tert-butyldimethylsilyloxy)methyl) pyrrolidin-3-ol (**83**) (39.40 g, 0.170 mol, 2.3 eq.) and anhydrous triethylamine (82.63 mL, 0.592 mol, 8 eq.) in anhydrous DCM (400 mL) at -40°C. The reaction mixture was allowed to slowly warm to room temperature (over 2.5 hours) after which, LCMS analysis indicated complete reaction. DCM (250 mL) was added and the mixture was transferred into a separating funnel. The organic layer was washed successively with 0.1M HCl (2 x 800 mL), saturated $NaHCO_3$ (500 mL) and brine (300 mL). After drying

over $MgSO_4$ and filtration, evaporation of the solvent left the product as a yellow foam (62.8 g, 92%). LC/MS, System 1: RT 1.96 min; MS (ES+) *m/z* (relative intensity) 921.45 ($[M+H]^+$, 100).

*(b) (5S,5'S)-1,1'-(4,4'-(pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitrobenzoyl))bis(5-(((tert-butyldimethylsilyl)oxy)methyl)pyrrolidin-3-one) (**85**)*

[0560] Trichloroisocyanuric acid (21.86 g, 94.07 mmol, 1.4 eq) was added in one portion to a solution of diol **84** (61.90 g, 67.20 mmol) and TEMPO (2.10 g, 13.44 mmol, 0.2 eq) in anhydrous DCM (500 mL) under an atmosphere of argon at 0°C. The reaction mixture was stirred at 0°C for 20 minutes after which, LCMS analysis of the reaction mixture showed complete reaction. The reaction mixture was diluted with DCM (400 mL) and washed with saturated sodium bicarbonate (500 mL), 0.2 M sodium thiosulfate solution (600 mL), brine (400 mL) and dried ($MgSO_4$). Evaporation of the solvent gave the crude product. Flash chromatography [gradient elution 80% n-hexane/20% ethyl acetate to 100% ethyl acetate] gave pure **85** as yellow solid (49.30 g, 80%). LC/MS, System 1: RT 2.03 min; MS (ES+) *m/z* (relative intensity) 917.55 ($[M+H]^+$, 100).

*(c) (5S,5'S)-1,1'-(4,4'-(pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitrobenzoyl))bis(5-(((tert-butyldimethylsilyl)oxy)methyl)-4,5-dihydro-1H-pyrrole-3,1-diyl) bis(trifluoromethanesulfonate), (**86**)*

[0561] Triflic anhydride (24.19 mL, 0.144 mol, 6.0 eq) was added dropwise to a vigorously stirred solution of bis-ketone **85** (21.98 g, 23.96 mmol) in anhydrous DCM (400 mL) containing 2,6-lutidine (22.33 mL, 0.192 mol, 8.0 eq) at -40 °C. The reaction mixture was stirred at -40 °C for 30 min after which, LCMS analysis indicated complete reaction. Reaction mixture was rapidly diluted with DCM (500 mL) and washed with ice-cold water (600 mL), ice-cold saturated sodium bicarbonate (400 mL) and brine (500 mL), dried over $MgSO_4$, filtered and evaporated to leave a crude brown oil. Flash chromatography [gradient elution 80% n-

hexane/20% ethyl acetate to 66% n-hexane/33% ethyl acetate] gave pure **86** as a brown foam (16.40 g, 58%). LC/MS, System 1: RT 2.28 min; MS (ES+) *m/z* (relative intensity) no data.

*(d) (S)-((pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitro-4,1-phenylene))bis(((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone) (**87**)*

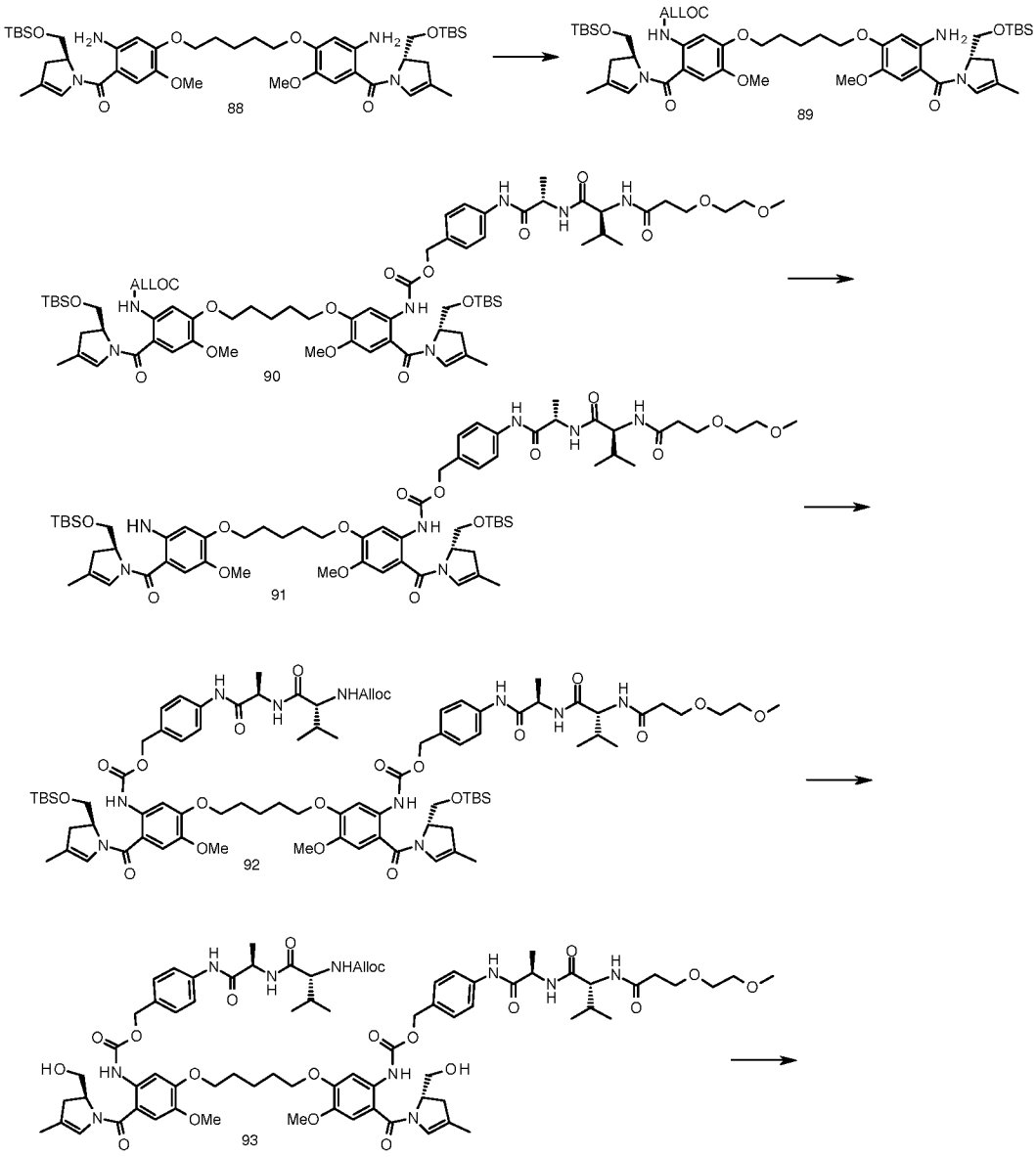
[0562] Triflate **86** (5.06 g, 4.29 mmol), methyl boronic acid (1.80 g, 30.00 mmol, 7 eq) and triphenylarsine (1.05 g, 3.43 mmol, 0.8 eq) were dissolved in anhydrous dioxane and stirred under argon. Pd (II) bisbenzonitrile chloride was then added and the reaction mixture heated rapidly to 80 °C for 20 min. Reaction mixture cooled, filtered through Celite (washed through with ethyl acetate), filtrate washed with water (500 mL), brine (500 mL), dried over $MgSO_4$, filtered and evaporated. Flash chromatography [gradient elution 50% n-hexane/50% ethyl acetate] gave pure **87** as a brown foam (4.31 g, 59%). LC/MS, System 1: RT 2.23 min; MS (ES+) *m/z* (relative intensity) 913.50 ($[M+H]^+$, 100).

*(e) (S)-((pentane-1,5-diylbis(oxy))bis(2-amino-5-methoxy-4,1-phenylene))bis(((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone) (**88**)*

[0563] Zinc dust (26.48 g, 0.405 mol, 36.0 eq) was added in one portion to a solution of bis-nitro compound **87** (10.26 g, 11.24 mmol) in 5% formic acid / methanol (200 mL) keeping the temperature between 25-30°C with the aid of a cold water bath. The reaction was stirred at 30°C for 20 minutes after which, LCMS showed complete reaction. The reaction mixture was filtered through Celite to remove the excess zinc, which was washed with ethyl acetate (600 mL). The organic fractions were washed with water (500 mL), saturated sodium bicarbonate (500 mL) and brine (400 mL), dried over $MgSO_4$ and evaporated. Flash chromatography [gradient elution 100% chloroform to 99% chloroform/1% methanol] gave pure **88** as an orange

foam (6.22 g, 65%). LC/MS, System 1: RT 2.20 min; MS (ES+) *m/z* (relative intensity) 853.50 ($[M+H]^+$, 100).

(ii) 4-((R)-2-((R)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl 4-((10R,13R)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl ((S)-(pentane-1,5-diylbis(oxy))bis(2-((S)-2-(hydroxymethyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5,1-phenylene))dicarbamate (93)



*(a) Allyl (5-((5-(5-amino-4-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-2-methoxyphenoxy)pentyl)oxy)-2-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxyphenyl)carbamate (**89**)*

[0564] Pyridine (1.156 mL, 14.30 mmol, 1.5 eq) was added to a solution of the bis-aniline **88** (8.14 g, 9.54 mmol) in anhydrous DCM (350 mL) at -78°C under an atmosphere of argon. After 5 minutes, allyl chloroformate (0.911 mL, 8.58 mmol, 0.9 eq) was added and the reaction mixture allowed to warm to room temperature. The reaction mixture was diluted with DCM (250 mL), washed with saturated $CuSO_4$ solution (400 mL), saturated sodium bicarbonate (400 mL) and brine (400 mL), dried over $MgSO_4$. Flash chromatography [gradient elution 66% n-hexane/33% ethyl acetate to 33% n-hexane/66% ethyl acetate] gave pure **89** as an orange foam (3.88 g, 43%). LC/MS, System 1: RT 2.27 min; MS (ES+) *m/z* (relative intensity) 937.55 ($[M+H]^+$, 100).

*(b) Allyl 4-((10S,13S)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl ((S)-(pentane-1,5-diylbis(oxy))bis(2-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5,1-phenylene))dicarbamate (**90**)*

[0565] Triethylamine (0.854 mL, 6.14 mmol, 2.2 eq) was added to a stirred solution of the aniline **89** (2.62 g, 2.79 mmol) and triphosgene (0.30 g, 1.00 mmol, 0.36 eq) in anhydrous THF (50 mL) under argon 0°C. The reaction mixture was stirred at room temperature for 5 minutes. LCMS analysis of an aliquot quenched with methanol, showed formation of the isocyanate. A solution of $mPEG_2$-Val-Ala-PAB-OH (1.54 g, 3.63 mmol, 1.3 eq) and triethylamine (0.583 mL, 4.19 mmol, 1.5 eq) in dry THF (50 mL) was added in one portion and the resulting mixture was stirred overnight at 40°C. The solvent of the reaction mixture was evaporated leaving a crude product. Flash chromatography [gradient elution 100% chloroform to

98% chloroform/2% methanol] gave pure **90** as a light orange solid (2.38 g, 62%). LC/MS, System 1: RT 2.29 min; MS (ES+) *m/z* (relative intensity) no data.

*(c) 4-((10S,13S)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl (5-((5-(5-amino-4-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-2-methoxyphenoxy)pentyl)oxy)-2-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxyphenyl)carbamate (**91**)*

[0566] Tetrakis(triphenylphosphine)palladium (39 mg, 0.034 mmol, 0.02 eq) was added to a stirred solution of **90** (2.35 g, 1.69 mmol) and pyrrolidine (0.35 mL, 4.24 mmol, 2.5 eq) in anhydrous DCM (25 mL) under argon at room temperature. Reaction mixture allowed to stir for 45 min then diluted with DCM (100 mL), washed with saturated ammonium chloride solution (100mL), brine (100mL), dried over $MgSO_4$, filtered and evaporated. Flash chromatography [gradient elution 100% chloroform to 95% chloroform/5% methanol] gave pure **91** as a yellow solid (1.81 g, 82%). LC/MS, System 1: RT 2.21 min; MS (ES+) *m/z* (relative intensity) 1303.65 ($[M+H]^+$, 100).

*(d) 4-((R)-2-((R)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl 4-((10R,13R)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl ((S)-(pentane-1,5-diylbis(oxy))bis(2-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5,1-phenylene))dicarbamate (**92**)*

[0567] Triethylamine (0.419 mL, 3.01 mmol, 2.2 eq) was added to a stirred solution of the aniline **91** (1.78 g, 1.37 mmol) and triphosgene (0.15 g, 0.49 mmol, 0.36 eq) in anhydrous THF (50 mL) under argon 0 °C. The reaction mixture was stirred at room temperature for 5 min. LCMS analysis of an aliquot quenched with methanol, showed formation of the isocyanate. A solution of Alloc-Val-Ala-PAB-OH (0.67 g, 1.78 mmol, 1.3 eq) and triethylamine (0.29 mL,

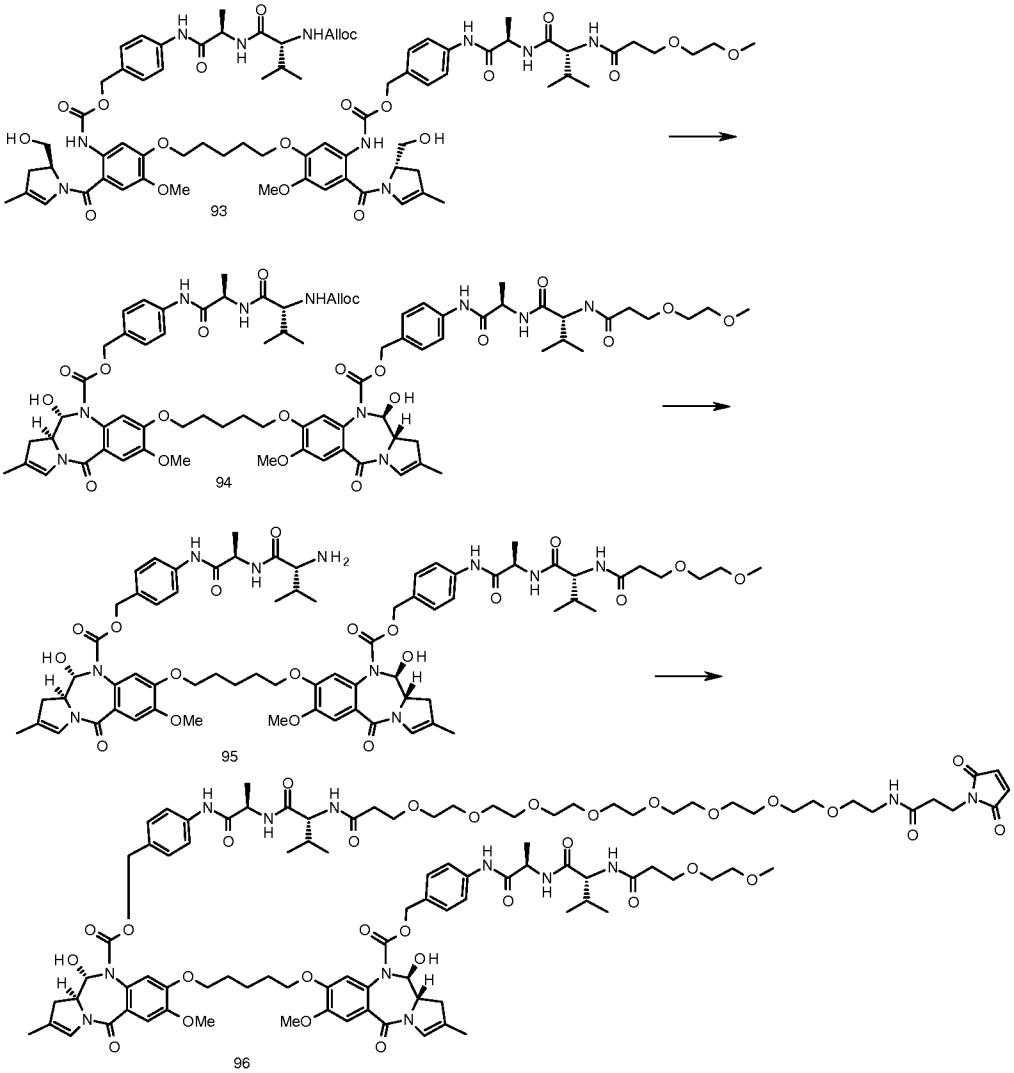
2.05 mmol, 1.5 eq) in dry THF (45 mL) was added in one portion and the resulting mixture was stirred overnight at 40 °C. The solvent of the reaction mixture was evaporated leaving a crude product. Flash chromatography [gradient elution 100% ethyl acetate to 97% ethyl acetate/3% methanol] gave pure **92** as a pale yellow solid (1.33 g, 57%).

[0568] LC/MS, System 1: RT 2.21 min; MS (ES+) *m/z* (relative intensity) no data.

*(e) 4-((R)-2-((R)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl 4-((10R,13R)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl ((S)-(pentane-1,5-diylbis(oxy))bis(2-((S)-2-(hydroxymethyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-5,1-phenylene))dicarbamate (**93**)*

[0569] Tetra-n-butylammonium fluoride (1 M, 1.52 mL, 1.52 mmol, 2.0 eq) was added to a solution of the TBS protected compound **92** (1.30 g, 0.76 mmol) in anhydrous THF (15 mL). The reaction mixture was stirred at room temperature for 4 hours. The reaction mixture was diluted with chloroform (100 mL) and washed sequentially with water (40 mL) and brine (40 mL). The organic phase was dried over $MgSO_4$ and evaporated to leave a yellow solid. Flash chromatography [gradient elution 95% ethyl acetate/5% methanol to 90% ethyl acetate/10% methanol] gave pure **93** as a pale yellow solid (1.00 g, 89%). LC/MS, System 1: RT 1.60 min; MS (ES+) *m/z* (relative intensity) 1478.45 (100).

(iii) (11S,11aS)-4-((2R,5R)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl 11-hydroxy-8-((5-(((11S,11aS)-11-hydroxy-10-(((4-((10R,13R)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl)oxy)carbonyl)-7-methoxy-2-methyl-5-oxo-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)pentyl)oxy)-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate (96)



*(a) (11S,11aS)-4-((R)-2-((R)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl 11-hydroxy-8-((5-(((11S,11aS)-11-hydroxy-10-(((4-((10R,13R)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl)oxy)carbonyl)-7-methoxy-2-methyl-5-oxo-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)pentyl)oxy)-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate (**94**)*

[0570] Dess-Martin periodinane (0.59 g, 1.38 mmol, 2.1 eq) was added to a stirred solution of **93** (0.97 g, 0.66 mmol) in anhydrous DCM under argon at room temperature. The

reaction mixture was allowed to stir for 4 hours. Reaction mixture diluted with DCM (100 mL), washed with saturated sodium bicarbonate solution (3 x 100 mL), water (100 mL), brine (100 mL), dried over $MgSO_4$, filtered and evaporated. Flash chromatography [gradient elution 100% chloroform to 95% chloroform/5% methanol] gave pure **94** as a pale yellow solid (0.88 g, 90%). LC/MS, System 1: RT 1.57 min; MS (ES+) *m/z* (relative intensity) 1473.35 (100).

*(b) (11S,11aS)-4-((R)-2-((R)-2-amino-3-methylbutanamido)propanamido)benzyl 11-hydroxy-8-((5-(((11S,11aS)-11-hydroxy-10-(((4-((10R,13R)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl)oxy)carbonyl)-7-methoxy-2-methyl-5-oxo-5,10,11,11a-tetrahydro-1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)pentyl)oxy)-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate (**95**)*

[0571] Tetrakis(triphenylphosphine)palladium (5 mg, 0.004 mmol, 0.06 eq) was added to a solution of **94** (105 mg, 0.071 mmol) and pyrrolidine (7 µL, 0.086 mmol, 1.2 eq) in anhydrous DCM (5 mL). The reaction mixture was stirred 15 minutes then diluted with chloroform (50 mL) and washed sequentially with saturated aqueous ammonium chloride (30 mL) and brine (30mL). The organic phase was dried over magnesium sulphate, filtered and evaporated. Flash chromatography [gradient elution 100% chloroform to 90% chloroform/10% methanol] gave pure **95** as a pale yellow solid (54 mg, 55%). LC/MS, System 1: RT 1.21 min; MS (ES+) *m/z* (relative intensity) 1389.50 (100).

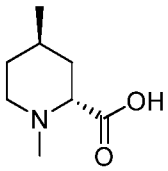
[0572] (c) (11S,11aS)-4-((2R,5R)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl 11-hydroxy-8-((5-(((11S,11aS)-11-hydroxy-10-(((4-((10R,13R)-10-isopropyl-13-methyl-8,11-dioxo-2,5-dioxa-9,12-diazatetradecanamido)benzyl)oxy)carbonyl)-7-methoxy-2-methyl-5-oxo-5,10,11,11a-tetrahydro-

1H-pyrrolo[2,1-c][1,4]benzodiazepin-8-yl)oxy)pentyl)oxy)-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate (**96**)

[0573] N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (28 mg, 0.146 mmol, 1 eq) was added to a solution of **95** (203 mg, 0.146 mmol) and maleimide-PEG_8 acid (87 mg, 0.146 mmol) in chloroform (5 mL). The reaction was stirred for 1.5 h then diluted with chloroform (50 mL), washed with water (50 mL), brine (30 mL), dried over magnesium sulphate, filtered and evaporated. Flash chromatography [gradient elution 100% DCM to 90% DCM/10% methanol] gave **96** as a pale yellow solid (205 mg, 72%). LC/MS, System 1: RT 5.75 min; MS (ES+) *m/z* (relative intensity) 982.90 (100), 1963.70 (5).

Example 21

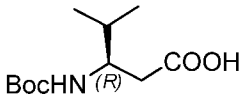
Compound **T1**



[0574] To a solution of (2R,4R)-4-methylpiperidine-2-carboxylic acid (2 g, 13.97 mmol) in MeOH (40 mL) and water (40.0 mL) was added paraformaldehyde (2.52 g, 27.94 mmol) and Pd/C (10%) (0.8 g, 7.52 mmol). The reaction mixture was stirred under a hydrogen atmosphere at room temperature overnight. From TLC, the reaction was not completed. Another one equivalent of paraformaldehyde (2.52 g, 27.94 mmol) was added and the reaction mixture was stirred another 24 hours. TLC indicated the reaction was completed and reaction mixture was filtered, washed the catalyst with MeOH (2 × 30 mL). The filtrate was concentrated *in vacuo* to give crude product as a white solid, which was washed with ether (3 × 30 mL), dried in high vacuum overnight to yield (2R,4R)-1,4-dimethylpiperidine-2-carboxylic acid (**T1**) (1.870 g, 85

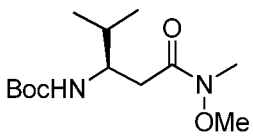
%) as a white solid. LC-MS: 158 (M+ 1); ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) δ ppm 0.97 (d, *J*=5.52 Hz, 3 H), 1.54 (br. s, 1 H), 1.71 - 1.87 (m, 3 H), 1.91 - 2.07 (m, 1 H), 2.84 (s, 3 H), 3.13 (td, *J*=8.41, 3.76 Hz, 1 H), 3.35 (m, 1 H), 3.65 (m, 1 H).

Compound **T2**



[0575] Boc_2O (243.0 g, 1.1 mol) was added dropwise to a suspension of (R)-3-amino-4-methyl pentanoic acid (commercially available) (133.0 g, 1.0 mol) and Na_2CO_3 (212 g, 2.0 mol) in acetone (1 L) and water (1 L) with stirring at room temperature. The reaction mixture was stirred overnight and the organic solvent was removed under reduced pressure. The residue was diluted with water (1 L) and washed with EtOAc (500 mL ×3). The aqueous phase was acidified with 2N HCl solution to pH=3 and the resulting mixture was extracted with EtOAc (800 mL ×3). The combined extracts were washed with brine (800 mL ×1), dried (Na_2SO_4) and concentrated to give compound (**T2**) (224.0 g, 97% yield) as an oil, which was used in the next step without further purification.

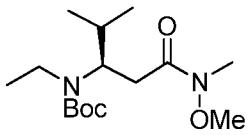
Compound **T3**



[0576] Triethylamine (67 g, 0.61 mol) was added to a suspension of compound (**2**) (140.0 g, 0.61 mol) and N,O-dimethylhydroamine hydrochloride (74.1 g, 0.76 mol) in CH_2Cl_2 (1.4 L) with stirring at 0°C. The suspension was stirred for 0.5 hour and EDCI (74 g, 0.61 mol) was added in portions at 0 °C. The reaction mixture was stirred for 2 hours at 0°C and water (800

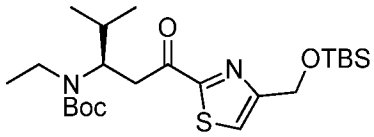
mL) was added. The organic phase was separated, washed with 5% KHSO4 solution (800 mL×3), saturated NaHCO3 solution (800 mL×3) and brine (800 mL×1), dried (Na2SO4) and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (EtOAc/Hexane=1:5) to afford compound (**3**) (141.0 g, 84% yield) as an oil. 1H NMR (300 MHz, CDCl3): δ 5.26 (m, 1H), 3.75 (m, 1H), 3.70 (s, 3H), 3.15 (s, 3H), 2.60~2.80 (m, 2H), 1.85 (m, 1H), 1.41 (s, 9H), 0.90 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H).

Compound **T4**



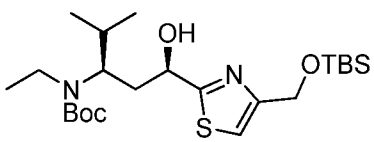
[0577] Iodoethane (250.0 g, 1.6 mol) was added to a solution of compound (**3**) (55.0 g, 0.2 mol) in DMF (1.1 L) with stirring at 0°C. Then NaH (60% suspension, 24.0 g, 0.60 mol) was added in portions at 0°C and the reaction mixture was allowed to warm to room temperature and stirred for 12 hours. The reaction was quenched with water (2 L) carefully and EtOAc (2 L) was added. The organic phase was separated, washed with 5% KHSO4 solution (800 mL×3), saturated NaHCO3 solution (800 mL×3) and brine (800 mL×1), dried (Na2SO4) and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (EtOAc/Hexane=1:10) to afford compound (**T4**) (35.1 g, 58% yield) as an oil. 1H NMR (300 MHz, CDCl3): δ 3.70 (s, 3H), 3.65 (m, 1H), 3.10~3.30 (m, 5H), 2.50~2.95 (m, 2H), 1.90~2.20 (m, 1H), 1.40~1.55 (m, 9H), 1.10 (t, J = 7.2 Hz, 3H), 0.90 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H).

Compound **T5**



[0578] A solution of n-BuLi (106 ml, 2.5N in hexane, 0.17 mol) was added dropwise to a solution of 2-bromo-4-((tert-butyldimethylsilyloxy)methyl)thiazole (74 g, 0.24 mol) (prepared as described in Wipf, P et al Org. Lett. 2007, 9(8), p. 1605) in dry THF (500 mL) at -78°C under N2 with stirring over 1 hour. The suspension was stirred for further 30 min and then a solution of compound (**T4**) (51.0 g, 0.17 mol) in dry THF (300 mL) was added dropwise over 30 min at -78°C. The reaction mixture was stirred for 1 hour at -78°C and then allowed to warm to room temperature and stirred for 12 hours. The reaction was quenched with 20% aqueous ammonium chloride solution (1 L) and the organic solvent was removed under reduced pressure. The resulting mixture was extracted with EtOAc (800 mL×3). The combined organic phases were washed with 5% KHSO4 solution (800 mL×3), saturated NaHCO3 solution (800 mL×3) and brine (800 mL×1), dried (Na2SO4) and concentrated to dryness. The crude material was purified by flash column chromatography on silica gel (EtOAc/Hexane=1:10) to afford compound (**5**) (58.1 g, 73% yield) as an oil. 1H NMR (300 MHz, CDCl3): δ 7.53 (m, 1H), 4.90 (s, 2H), 4.04 (m, 1H), 3.35 (m, 2H), 3.15 (m, 2H), 2.00 (m, 1H), 1.40 (s, 9H), 0.80~1.20 (m, 21H), 0.14 (s, 6H).

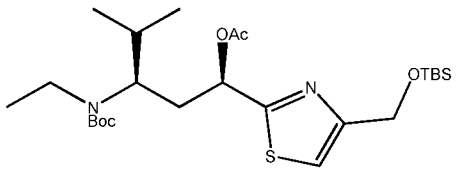
Compound **T6**



[0579] LiBH4 (4.8 g, 0.22 mol) was added in portions to a solution of compound (**T5**) (47.1 g, 0.1 mol) in methanol (500 mL) at room temperature over a period of 0.5 hour with

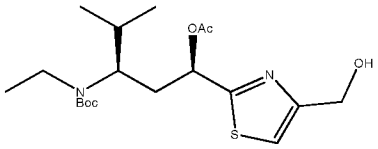
stirring. The suspension was stirred for 2 hours and the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (800 mL) and the resulting solution was washed with saturated NaHCO3 solution (500 mL×3) and brine (500 mL×1), dried (Na2SO4) and concentrated to dryness. The crude material was purified by flash column chromatography (EtOAc/Hexane=1:6) to afford compound (**T6**) (13.5 g, 28% yield) and its isomer (**T6'**) (21.0 g, 45% yield). ^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm -0.06 - 0.05 (m, 6 H) 0.76 - 0.89 (m, 15 H) 1.12 (t, J=6.97 Hz, 3 H) 1.39 (s, 9 H) 1.55 - 2.05 (m, 3 H) 2.86 - 3.21 (m, 2 H) 3.76 - 3.96 (m, 1 H) 4.73 (d, J=1.13 Hz, 4 H) 7.01 (s, 1 H).

Compound **T7**



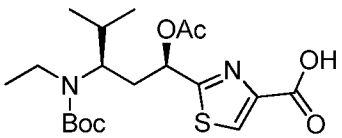
[0580] Acetyl chloride (45.2 g, 0.58 mol) was added dropwise to a solution of compound (**T6**) (34.0 g, 72 mmol) in pyridine (500 mL) at 0°C with stirring over 10 min. The reaction mixture was allowed to warm to room temperature and stirred for 12 hours. The reaction was quenched with water (200 mL) and the solvent was removed under reduced pressure. The residue was treated with CH_2Cl_2 (800 mL) and the resulting mixture was washed with 5% $KHSO_4$ solution (800 mL×3), saturated $NaHCO_3$ solution (800 mL×3) and brine (800 mL×1), dried (Na_2SO_4) and concentrated to dryness. The crude material was purified by flash column chromatography on silica gel (EtOAc/ Hexane=1:10) to afford compound (**7**) (25.7 g, 69% yield) as an oil. ^{1}H NMR (300 MHz, CDCl3): δ 7.15 (m, 1H), 5.95 (m, 1H), 4.84 (s, 2H), 4.04 (m, 1H), 3.10 (m, 2H), 2.35 (m, 1H), 2.15 (s, 3H), 2.00 (m, 1H), 1.70 (m, 1H), 1.45 (s, 9H), 1.25 (t, J = 7.2 Hz, 3H), 0.80~1.10 (m, 21H), 0.08 (s, 6H).

Compound **T8**



[0581] A solution of tetrabutylammonium fluoride (65.3 g, 0.25 mol) in THF (200 mL) was added dropwise to a solution of compound (**7**) (25.7 g, 50 mmol) in THF (300 mL) at 0°C with stirring. The reaction mixture was allowed to warm to room temperature and stirred for 4 hours. Water (800 mL) was added and the organic solvent was removed under reduced pressure. The residue was treated with CH_2Cl_2 (800 mL) and the resulting mixture was washed with 5% $KHSO_4$ solution (800 mL×3), saturated $NaHCO_3$ solution (800 mL×3) and brine (800 mL×1), dried (Na2SO4) and concentrated to dryness. The crude material was purified by flash column chromatography on silica gel (EtOAc/ Hexane=1:4) to afford compound (**8**) (19.5 g, 98% yield) as an oil. 1H NMR (300 MHz, CDCl3): δ 8.26 (m, 1H), 5.95 (m, 1H), 4.10 (m, 1H), 3.17 (m, 2H), 2.40 (m, 1H), 2.20 (s, 3H), 2.18 (m, 1H), 1.75 (m, 1H), 1.56 (s, 9H), 1.10~1.30 (m, 4H), 0.80~1.05 (m, 6H).

Compound **T9**

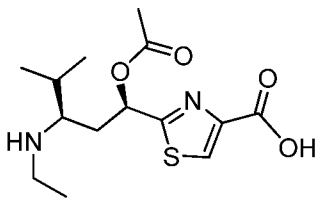


[0582] Dess-Martin reagent (32.7 g, 75 mmol) was added to a solution of compound (**T8**) (20.0 g, 50 mmol) in dichloromethane (300 mL) and the reaction mixture was stirred at room temperature for 12 hours. The mixture was washed with sodium hydroxide solution (1N, 300 mL×3), sodium thiosulfate solution (1N, 300 mL×3), saturated $NaHCO_3$ (300 mL×3) solution

and brine (300 mL×1), respectively. The organic layer was dried (Na_2SO_4) and concentrated to dryness to give the corresponding aldehyde.

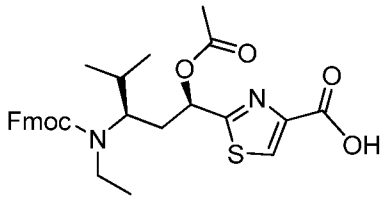
[0583] This crude aldehyde was dissolved in tert-butyl alcohol (500 mL) and a solution of sodium chlorite (80%, 36.4 g, 320 mmol) and sodium dihydrogenphosphate monohydrate (105 g, 0.77 mol) in water (300 mL) was added dropwise over 1 hour at room temperature. The reaction mixture was stirred for 3 hours and diluted with hydrochloric acid solution (0.1N, 500 mL). The resulting mixture was extracted with EtOAc (500 mL×1) and the combined organic layers were washed with 5% $KHSO_4$ solution (500 mL×3) and brine (500 mL×1), dried over Na_2SO_4 and concentrated to dryness. The residue was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH=100:5) to afford compound (**T9**) (15.4 g, 58% yield). 1H NMR (300 MHz, CDCl3): δ 9.90 (br s, 1H), 8.27 (s, 1H), 5.96 (m, 1H), 4.07 (m, 1H), 3.15 (m, 1H), 2.35 (m, 1H), 2.20 (s, 3H), 2.18 (m, 1H), 1.75 (m, 1H), 1.45 (s, 9H), 1.20 (t, J = 7.2 Hz, 3H), 0.98 (d, J = 6.6 Hz, 3H), 0.88 (d, J = 6.6 Hz, 3H).

Compound **T10**



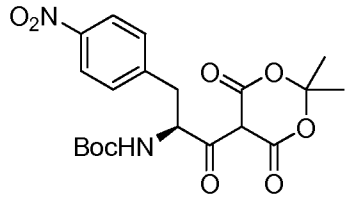
[0584] To a solution of 2-((1R,3R)-1-acetoxy-3-((tert-butoxycarbonyl)(ethyl)amino)-4-methylpentyl)thiazole-4-carboxylic acid **9** (6.5g, 15.68 mmol) in DCM (60 mL) was added TFA(30 mL) in dropwise at 0 °C. The mixture was stirred at 0 °C for 1 hour. The solvent was evaporated *in vacuo* to give crude product (**T10**). The crude product was used to next step reaction without further purification (7.2 grams). LC-MS : 315 (M + 1).

Compound **T11**



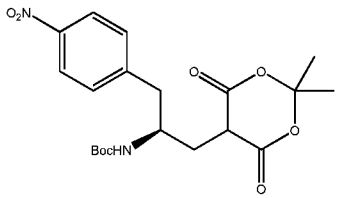
[0585] To the solution of 2-((1R,3R)-1-acetoxy-3-(ethylamino)-4-methylpentyl)thiazole-4-carboxylic acid 4, Trifluoroacetic acid salt (**T10**) (5g, 11.67 mmol) and sodium bicarbonate (9.80 g, 116.71 mmol) in a mixture of acetone (300 mL) and water (150 mL) was added (9H-fluoren-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (3.94 g, 11.67 mmol). The mixture was stirred at room temperature overnight. LCMS indicated the reaction was completed. The mixture was acidified to (pH 2) with hydrochloric acid and acetone was evaporated *in vacuo*. The product was extracted with DCM (3 × 300 mL). The combined organic extracts were washed with 0.1% HCl solution (200 mL), brine (200 mL), dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by flash chromatography (silica gel, MeOH/DCM, MeOH from 0% to 5%,) to give 2-((1R,3R)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)(ethyl)amino)-1-acetoxy-4-methylpentyl)thiazole-4-carboxylic acid (**T11**) (3.53 g, 54.6 %) as a white solid. LC-MS : 537.2 (M+1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 0.84 (d, *J*=6.78 Hz, 3 H), 0.92 - 1.05 (m, 5 H), 1.14 (d, *J*=3.01 Hz, 1 H), 1.73 (dt, *J*=10.23, 6.43 Hz, 1 H), 1.92 - 2.05 (m, 1 H), 2.12 - 2.27 (m, 4 H), 2.28 - 2.44 (m, 1 H), 2.90 - 3.33 (m, 3 H), 3.98 (t, *J*=9.29 Hz, 1 H), 4.12 - 4.32 (m, 1 H), 4.47 - 4.82 (m, 2 H), 5.95 (dd, *J*=10.92, 2.89 Hz, 1 H), 7.29 - 7.45 (m, 4 H), 7.55 - 7.69 (m, 2 H), 7.72 - 7.81 (m, 2 H), 8.22 - 8.29 (m, 1 H).

Compound **T12**



[0586] DMAP (106 g, 0.86 mol) was added to a solution of Boc-L-4-nitro-Phenylalanine (1800 g, 0.58 mol) and Meldrum's acid (92 g, 0.64 mol) in dichloromethane (1.5 L). The resulting solution was cooled at -5°C under N2 atmosphere, followed by addition of DCC (240 g, 1.16 mol) in dichloromethane (1 L) over 1 h. The mixture was stirred overnight at 0~5°C. Then the precipitated N,N'-dicyclohexylurea was removed by filtration and the filtrate was washed with 5% aqueous HCl (1 L × 3), and brine (1 L × 1), and was dried over MgSO4. After removal of MgSO4 by filtration, the organic phase was concentrated to dryness. The residue was triturated with EtOAc/hexane (1:1, 500 mL), and was dried to afford compound (**T12**) (130.0 g, 51% yield) as a yellow solid.

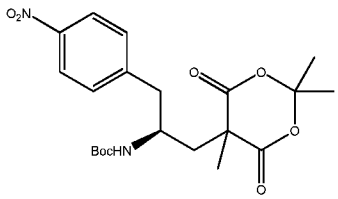
Compound **T13**



[0587] AcOH (400 mL) was added to a solution of compound (**T12**) (130.0 g, 0.298 mol) in dichloromethane (1.5 L) at -5°C under N2. Solid $NaBH_4$ (22.7 g, 0.597 mol) was added in small portions over 2 hours (gas evolution and exothermic). After stirring for additional 3 h at -5 °C, TLC indicated the reaction was complete. The mixture was quenched with brine (1 L). The organic layer was separated, and washed sequentially with water (1 L×2), aqueous saturated NaHCO3 (1 L×3) and brine (1 L×3), and was dried over MgSO4. The filtrate was concentrated to dryness and afford compound (**T13**) (70.3 g, 55 % yield) as a yellow solid. 1H NMR (300

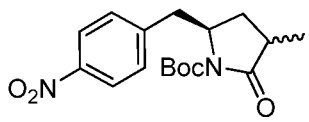
MHz, CDCl3): δ 8.18 (d, J=8.7 Hz, 2H), 7.41 (d, J=8.7 Hz, 2H), 4.58 (m, 1H), 4.29 (m, 1H), 3.85 (m,1H), 2.97 (d, J=6.6 Hz, 2H), 2.27 (m ,2H), 1.80 (s, 3H), 1.76 (s, 3H), 1.35 (s, 9H).

Compound **T14**



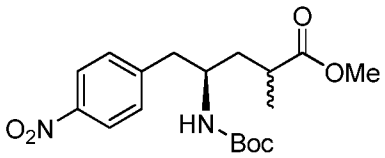
[0588] K_2CO_3 (35 g, 0.25 mol) and MeI (36 g, 0.25 mol) were added to a solution of compound (**T13**) (70.3 g, 0.167 mol) in acetone (400 mL) and DMF (400 mL). The mixture was stirred overnight at room temperature. TLC showed the starting material was consumed. Water (2 L) was added and the mixture was stirred for an additional hour. The precipitated solid was collected by filtration, was washed with water, was dried to afford compound (**T14**) (34.5 g, 47% yield) as a pale yellow solid. 1H NMR (300 MHz, CDCl3): δ 8.17 (d, J=8.7 Hz, 2H), 7.34 (d, J=8.7 Hz, 2H), 4.22 (m, 1H), 3.85 (m,1H), 2.85 (m, 2H), 2.22 (m ,2H), 1.73 (s, 3H), 1.73 (s, 3H), 1.52 (s, 3H), 1.31 (s, 9H).

Compound **T15**



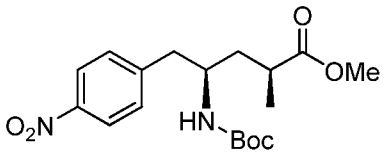
[0589] Compound (**T14**) (34.5 g, 79.1 mmol) was dissolved in toluene (500 mL). The solution was heated under reflux for 40 hours. TLC indicated the reaction complete. The solvent was removed to afford compound (**T15**) (30g), which was used for next step without further purification.

Compound T16



[0590] K_2CO_3 (22 g, 0.16 mol) was added to a solution of compound (**T15**) (30 g, 79 mmol) in MeOH (300 mL). The mixture was stirred for 3 hours at room temperature. TLC showed complete conversion. The solvent was removed, the residue was dissolved in dichloromethane (500 mL), washed with brine (500 mL×3), dried over $MgSO_4$. After removal of $MgSO_4$ by filtration, the organic phase was concentrated to dryness. The residue was further purified by silica gel chromatography (EtOAc/Hexane=1:10) and afforded compound (**T16**) (23.5 g, 81 % yield for two steps) as 1:1 diastereomeric mixture. ^{1}H NMR (300 MHz, CDCl3): δ 8.13 (d, J=8.7 Hz, 2H), 7.34 (d, J=8.7 Hz, 2H), 4.43 (m, 1H), 3.85 (m,1H), 3.65 (s ,3H), 2.85 (m, 2H), 2.65 (m ,1H), 1.85 (m, 1H), 1.50 (m, 1H), 1.30 (s, 9H), 1.15 (t, J=6.6 Hz, 6H).

Compound **T17**



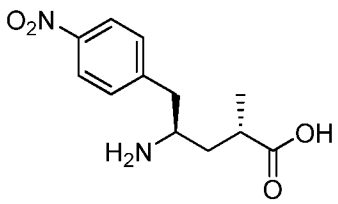
[0591] 50 g of compound (**T16**) was subjected to chiral chromatography using SFC (supercritical fluid chromatography) on a Chiralpak ID 21×250 mm, 5 μ column using mobile phase A 90% carbon dioxide and phase B isopropanol 10% at a 60 ml/min flow rate. The separation was performed at 40 °C and detection at 270 nM. Baseline separation was achieved and two fractions were isolated. Peak B was desired compound (**T17**) and was obtained as a solid 27.4 g (55%).

[0592] >99:1 diastereomeric excess on a Chiralpak IA column 4.6×250 mm, 5μ, 10% 1:1 Methanol:Isopropanol in hexane with 0.1% diethylamine modifier.

[0593] LC/MS (2 minute, Acid _CV10.olp method 367 (M + 1), 1.16 minutes.

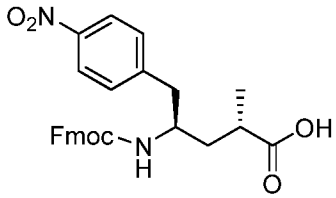
[0594] ^{1}H NMR (400 MHz, METHANOL-d_4) δ ppm 8.16 (d, J=8.53 Hz, 2 H) 7.46 (d, J=8.53 Hz, 2 H) 3.79 - 3.93 (m, 1 H) 3.68 (s, 3 H) 2.90 - 2.99 (m, 1H) 2.71 - 2.81 (m, 1 H) 2.47 - 2.59 (m, 1 H) 1.81 - 1.95 (m, 1 H) 1.55 - 1.66 (m, 1 H) 1.32 (s, 9 H) 1.21 - 1.25 (m, 2 H) 1.16 (d, J=7.03 Hz, 3 H).

Compound **T18**



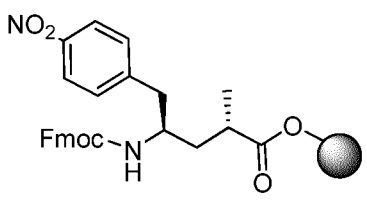
[0595] A solution of (2S,4R)-methyl 4-((tert-butoxycarbonyl)amino)-2-methyl-5-(4-nitrophenyl)pentanoate (**T17**) (3.5g, 9.55 mmol) in 6N HCl aqueous solution (8.0 mL, 263.30 mmol) was heated at 130 °C in microwave for 30 min. The reaction mixture was lyophilized to afford (2S,4R)-4-amino-2-methyl-5-(4-nitrophenyl)pentanoic acid (**T18**) as a solid. The product was used in the next step reaction without further purification. (3.2 g). LC-MS : 253 (M + 1); ^{1}H NMR (400 MHz, DEUTERIUM OXIDE) δ ppm 1.12 (d, *J*=7.28 Hz, 3 H), 1.62 - 1.76 (m, 1 H), 1.90 - 2.02 (m, 1 H), 2.56 - 2.68 (m, 1 H), 3.02 - 3.11 (m, 2 H), 3.58 - 3.69 (m, 1 H), 7.47 (d, *J*=8.53 Hz, 2 H), 8.18 (d, *J*=8.78 Hz, 2 H).

Compound **T19**



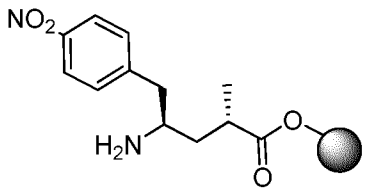
[0596] To the solution of compound (**T18)** (0.43 g, 1.49 mmol) and sodium bicarbonate (1.251 g, 14.89 mmol) in a mixture of acetone (30 mL) and water (15 mL) was added (9H-fluoren-9-yl)methyl 2,5-dioxopyrrolidin-1-yl carbonate (0.502 g, 1.49 mmol). The mixture was stirred at room temperature for overnight. LCMS indicated the reaction was completed. The mixture was acidified to pH 2 with hydrochloric acid and acetone was evaporated *in vacuo*. The product was extracted with DCM (3 X 60 mL). The combined organic extracts were washed with 1N HCl solution (40 mL), brine (40 mL), dried over Na_2SO_4, and evaporated *in vacuo.* The residue was purified by silica gel flash chromatography, EtOAc from 0% to 100% in DCM, to give (2S,4R)-4-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-methyl-5-(4-nitrophenyl)pentanoic acid (0.630 g, 89 %) (**T19**) as a white solid. LC-MS : 475.5 (M+H); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ ppm 0.81 - 1.06 (m, 1 H), 1.08 - 1.28 (m, 2 H), 1.33 - 1.75 (m, 1 H), 1.77 - 2.11 (m, 1 H), 2.36 - 2.69 (m, 2 H), 2.76 - 3.18 (m, 1 H), 3.43 - 4.08 (m, 1 H), 4.09 - 4.19 (m, 1 H), 4.21 - 4.53 (m, 2 H), 4.54 - 4.80 (m, 1 H), 7.18 - 7.58 (m, 8 H), 7.66 - 7.82 (m, 2 H), 7.95 - 8.17 (m, 2 H), 8.67 (br. s., 1 H.

Compound **T20**



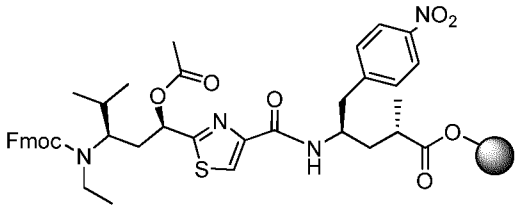
[0597] DIEA (0.419 mL, 2.40 mmol) was added to a solution of (2S,4R)-4-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-methyl-5-(4-nitrophenyl)pentanoic acid (0.380 g, 0.80 mmol) (**19**) in DCM (4.5 mL), and the mixture was stirred at room temperature for 5 min, then 2-Chlorotrityl chloride resin (0.5 g, 0.80 mmol) was added to the mixture. The mixture was shaken at room temperature overnight, the resulting resin was washed with DMF (3 X 6 mL), MeOH (3 X 6 mL), and DCM (3 X 6 mL), then treated with DIEA (0.419 mL, 2.40 mmol) and MeOH/DCM (1:1, 5 mL) at room temperature for 30 min. Resulting resin was filtered, washed with DMF (3 X 6 mL), MeOH (3 X 6 mL), and DCM (3 X 6 mL), dried in high vacuum overnight. Small amount of compound was cleaved from resin, and analyzed by LCMS. The resulting resin **(T20)** was used for next step reaction. LC/-MS : 475 (M + 1)

Compound **T21**



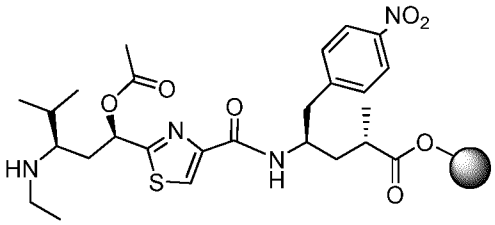
[0598] To the resin **(T20)** (0.5 g, 0.80 mmol) was added 20% piperidine in DMF (5 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin **(T21)** was used for the next step reaction. LC-MS: 253 (M + H)

Compound **T22**



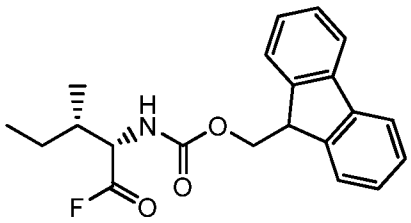
[0599] To the resin (**T21**) (0.5 g, 1.88 mmol) was added a solution of 2-((1R,3R)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)(ethyl)amino)-1-acetoxy-4-methylpentyl)thiazole-4-carboxylic acid (**3**) (1.108 g, 2.07 mmol), HATU (1.428 g, 3.76 mmol), 2,4,6-trimethylpyridine (0.500 mL, 3.76 mmol), and DIEA (0.656 mL, 3.76 mmol) in DMF (5 mL) at room temperature. The mixture was shaken at room temperature for two hours, and the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), and DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T22)** was used for the next step. LC-MS : 771 (M + H).

Compound **T23**



[0600] To the resin (**T22)** (0.5 g, 0.80 mmol) was added 20% piperidine in DMF (5 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T23)** was used in the next reaction step. LC-MS : 549 (M + 1).

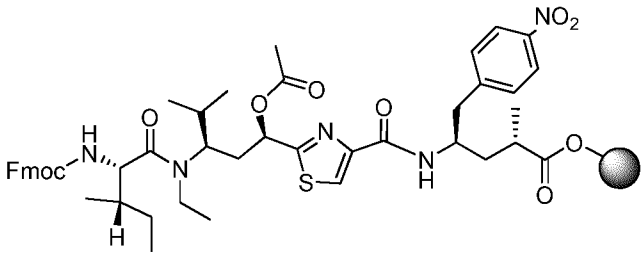
Compound **T24**



[0601] To a solution of (2S,3S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-3-methylpentanoic acid (Fmoc-Isoleucine) (7 g, 19.81 mmol) and pyridine (1.602 mL, 19.81 mmol) in DCM (120 mL) was added via cannula a solution of DAST (3.11 mL, 23.77 mmol) in DCM (20 mL) over 10 min. The reaction mixture was stirred at room temperature for 1 hour, diluted with DCM (80 mL), washed with ice-cold water (2 × 200 mL), the organic layer was dried over MgSO4, filtered, and evaporated in vacuo to give (9H-fluoren-9-yl)methyl (2S,3S)-1-fluoro-3-methyl-1-oxopentan-2-ylcarbamate (6.65 g, 94 %) as a white solid. An esterification test was performed to assure quantitative acid fluoride formation by dissolving Fmoc-Ile-F (5 mg) in anhydrous MeOH (0.3 mL) and DIEA (0,030 mL) and allowing to react at room temperature for 15 min. The mixture was then evaporated in vacuo and analyzed by LCMS, showed less than 1% of Fmoc-Ile-OH present.

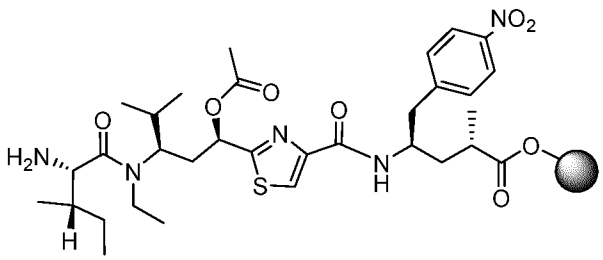
[0602] 1H NMR (400 MHz, CHLOROFORM-d) δ ppm 0.83 - 1.12 (m, 6 H) 1.18 - 1.37 (m, 1 H) 1.42 - 1.59 (m, 1 H) 2.01 (br. s., 1 H) 4.26 (t, J=6.78 Hz, 1 H) 4.44 - 4.63 (m, 3 H) 5.20 (d, J=8.53 Hz, 1 H) 7.31 - 7.39 (m, 2 H) 7.40 - 7.47 (m, 2 H) 7.61 (d, J=7.28 Hz, 2 H) 7.80 (d, J=7.53 Hz, 2 H)

Compound **T25**



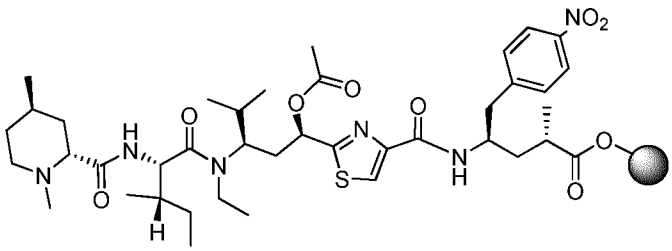
[0603] To the resin **(T23)** (0.5 g, 0.80 mmol) was added a solution of (9H-fluoren-9-yl)methyl (2S,3S)-1-fluoro-3-methyl-1-oxopentan-2-ylcarbamate (**T24**) (0.569 g, 1.60 mmol), DMAP (4.89 mg, 0.04 mmol), and DIEA (0.419 mL, 2.40 mmol) in DCM (5 mL) at room temperature. The mixture was shaken at room temperature for overnight, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried in high vacuum. Small amount of compound was cleaved from resin, and analyzed by LC/MS, LCMS indicated the reaction was completed. The resulting resin (**T25)** was used in the next reaction step. LC-MS : 884 (M + H).

Compound **T26**



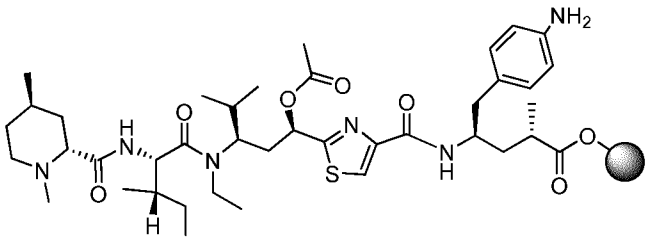
[0604] To the resin (**T25)** (0.5 g, 0.80 mmol) was added 20% piperidine in DMF (5 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin **(T26)** was used in the next reaction step. LC-MS : 662 (M + 1).

Compound **T27**



[0605] To the resin **(T26)** (0.5 g, 0.80 mmol) was added a solution of (2R,4R)-1,4-dimethylpiperidine-2-carboxylic acid **(1)** (0.252 g, 1.60 mmol), HATU (0.608 g, 1.60 mmol), 2,4,6-trimethylpyridine (0.320 mL, 2.40 mmol), and DIEA (0.419 mL, 2.40 mmol) in DMF (5 mL). The mixture was shaken at room temperature for 2 hours, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), and DCM (3 × 6 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T27)** was used in the next reaction step. LC-MS : 801 (M + 1).

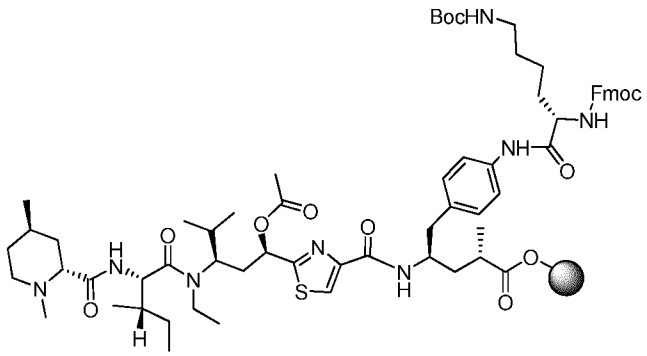
Compound **T28**



[0606] To the resin (**T27)** was added a solution of tin(II) chloride dehydrate (1.805 g, 8.00 mmol), and sodium acetate (0.197 g, 2.40 mmol) in DMF (5 mL). The mixture was shaken

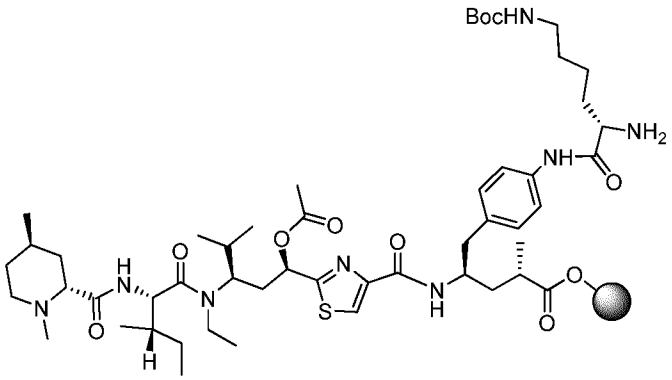
at room temperature for 4 hours, the resulting resin was filtered, washed with DMF (3 × 6 mL), MeOH (3 × 6 mL), and DCM (3 × 6 mL), and dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T28)** was used for the next step. LC-MS: 771 (M + H).

Compound **T29**



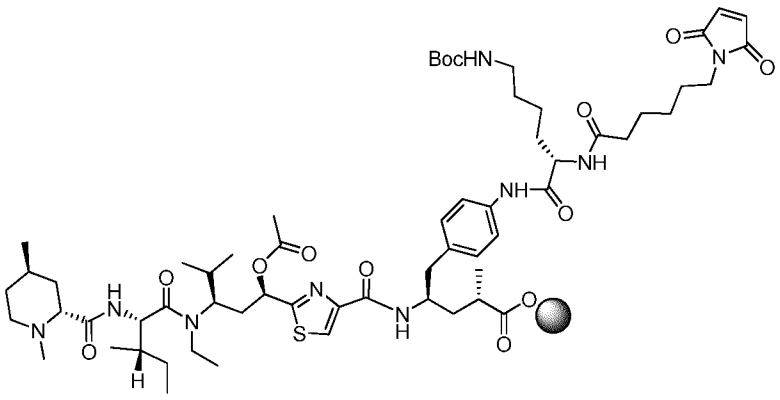
[0607] To the resin (**T28)** (0.2 g, 0.32 mmol) was added a solution of (S)-2-(((9H-fluoren-9-yl)methoxy)carbonylamino)-6-(tert-butoxycarbonylamino)hexanoic acid (commercially available) (0.300 g, 0.64 mmol), HATU (0.243 g, 0.64 mmol), 2,4,6-trimethylpyridine (0.128 mL, 0.96 mmol), and DIEA (0.168 mL, 0.96 mmol) in DMF (4 mL). The mixture was shaken at room temperature for 2 hours, the resulting resin was filtered, washed with DMF (3 × 2 mL), MeOH (3 × 2 mL), and DCM (3 × 2 mL), and dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T29)** was used to next step reaction. LC-MS: 1221 (M + 1).

Compound **T30**



[0608] To the resin (**T29)** (0.2 g, 0.32 mmol) was added 20% piperidine in DMF (2 mL). The mixture was shaken at room temperature for 6 min, the resulting resin was filtered, washed with DMF (3 × 3 mL), MeOH (3 × 3 mL), DCM (3 × 3 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T30)** was used to next step reaction. LC/MS : 999 (M + H).

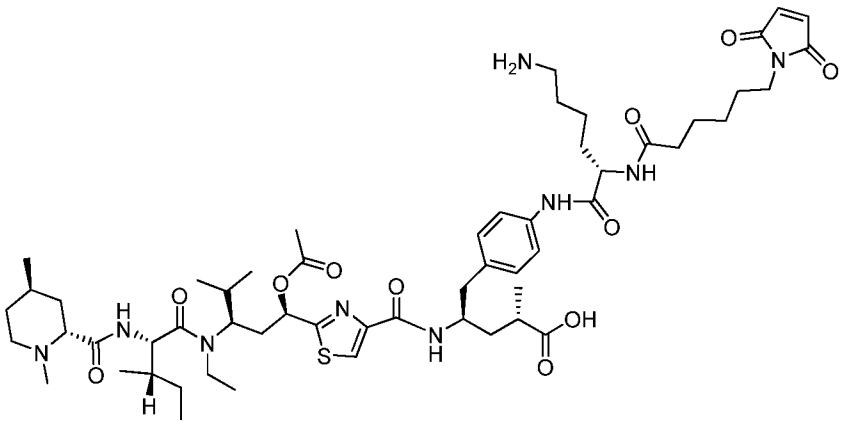
Compound **T31**



[0609] To the resin (**T30)** (0.2 g, 0.32 mmol) was added a solution of 2,5-dioxopyrrolidin-1-yl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate (0.148 g, 0.48 mmol) in DMF (2 mL) , followed by N-METHYLMORPHOLINE (0.106 mL, 0.96 mmol) at room temperature. The mixture was shaken at room temperature for 2 hours, the resulting resin was

filtered, washed with DMF (3 × 3 mL), DCM (3 × 3 mL), dried *in vacuo*. Small amount of the compound was cleaved from resin, analyzed by LCMS, which indicated the reaction was completed. The resulting resin (**T31)** was used for the next step. LC-MS: 1192 (M + 1).

Compound **T32** (also refeered to herein as 1508)



[0610] To the resin (**T31)** (0.2 g, 0.32 mmol) was added DCM (1 mL),and TFA (1 mL) at room temperature. The mixture was shaken at room temperature for 20 min, then filtered. The resin was washed with DCM/TFA (1:1, 3 × 2 mL), the filtrates were evaporated *in vacuo*. The residue was purified by reverse phase HPLC (ACN/H_2O (contained 0.1%TFA), ACN from 5% to 75% in 14 min.) The pure fractions were lyophilized to give (2S,4R)-4-(2-((1R,3R)-1-acetoxy-3-((2S,3S)-2-((2R,4R)-1,4-dimethylpiperidine-2-carboxamido)-N-ethyl-3-methylpentanamido)-4-methylpentyl)thiazole-4-carboxamido)-5-(4-((S)-6-amino-2-(6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanamido)hexanamido)phenyl)-2-methylpentanoic acid (**T32**) (0.095 g, 22.48 %) as a white solid. LC-MS : 1092 [M+1]; ^{1}H NMR (400 MHz, METHANOL-d4) δ ppm 7.99 (s, 1 H), 7.34 (d , *J* =8.53 Hz, 2 H), 7.10 (d , *J*=8.53 Hz, 2 H), 6.66 (s, 2 H), 5.64 (d, *J*=10.79 Hz, 1 H), 4.50 - 4.61 (m, 1 H), 4.21 - 4.35 (m, 2 H), 3.92 (d, *J*=9.29 Hz, 1 H), 3.69 (br. s., 1 H), 3.37 (t,

J=7.15 Hz, 2 H), 3.15-3.35 (m, 4H), 3.04 (dt, *J*=3.58, 1.85 Hz, 1 H), 2.84 (t, *J*=7.65 Hz, 2 H), 2.76 (d, *J*=7.03 Hz, 2 H), 2.62 (br. s., 2 H), 2.38 - 2.52 (m, 2 H), 2.25 (t, *J*=11.54 Hz, 1 H), 2.16 (t, *J*=7.40 Hz, 2 H), 2.04 - 2.11 (m, 4 H), 1.70 - 2.00 (m, 7 H) 1.42 - 1.69 (m, 11 H), 1.34 - 1.40 (m, 1 H), 1.27 (t, *J*=6.78 Hz, 3 H), 1.16 - 1.24 (m, 2 H), 1.01 - 1.14 (m, 7 H), 0.90 (d, *J*=6.78 Hz, 3 H), 0.94 (d, *J*=6.53 Hz, 3 H), 0.84 (t, *J*=7.40 Hz, 3 H), 0.79 (d, *J*=6.53 Hz, 3 H).

INCORPORATION BY REFERENCE

[0611] All references cited herein, including patents, patent applications, papers, text books, and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated herein by reference in their entirety for all purposes.

EQUIVALENTS

[0612] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the embodiments. The foregoing description and Examples detail certain embodiments and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the embodiments may be practiced in many ways and the claims include any equivalents thereof.

Chemical Abbreviations

ACN	acetonitrile
Ac	acetyl
Acm	acetamidomethyl
Alloc	allyloxycarbonyl
Boc	di-*tert*-butyl dicarbonate
Boc	t-Butoxycarbonyl
Bzl	benzyl, where Bzl-OMe is methoxybenzyl and Bzl-Me is methylbenzene
Cbz or Z	benzyloxy-carbonyl, where Z-Cl and Z-Br are chloro- and bromobenzyloxy carbonyl respectively
DAST	Diethylaminosulfur trifluoride
DCM	dichloromethane
DIAD	Diisopropyl azodicarboxylate
DIC	N,N'-diisopropylcarbodiimide
DIEA	diethylisopropylamine
DMA	N,N-dimethylacetamide
DMAP	Dimethylaminopyridine
DMF	N,N-dimethylformamide
Dnp	dinitrophenyl
DTT	dithiothreitol
Et2O	Diethylether;
EtOAc	Ethyl acetate;
Fmoc	9*H*-fluoren-9-ylmethoxycarbonyl
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxid hexafluorophosphate
HCl	Hydrochloric acid
imp	*N*-10 imine protecting group: 3-(2-methoxyethoxy)propanoate-Val-Ala-PAB
LC-MS	Liquid chromatography-mass spectrometry
MeOH	methanol
Moc	methoxycarbonyl
MP	maleimidopropanamide
Mtr	4-methoxy-2,3,6-trimethtylbenzenesulfonyl
Na_2CO_3	sodium bicarbonate
$NaHCO_3$	sodium hydrogen carbonate
MC-OSu	maleimidocaproyl-O-*N*-succinimide
PAB	para-aminobenzyloxycarbonyl
PEG	ethyleneoxy
PNZ	*p*-nitrobenzyl carbamate
Psec	2-(phenylsulfonyl)ethoxycarbonyl
RT	room temperature

TBDMS	tert-butyldimethylsilyl
TBDPS	tert-butyldiphenylsilyl
t-Bu	tert-butyl
TEA	triethylamine
Teoc	2-(trimethylsilyl)ethoxycarbonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Tos	tosyl
Troc	2,2,2-trichlorethoxycarbonyl chloride
Trt	trityl
Xan	xanthyl

WHAT IS CLAIMED IS:

1. A synthetic or recombinant antibody, or an antigen binding fragment thereof, capable of specifically binding 5T4 cell surface antigen comprising:

a. a heavy chain variable region CDR1 sequence comprising a sequence $GX_1X_2X_3X_4X_5X_6DX_7X_8X_9$, wherein
X_1=F, D or E,
X_2=T, W or G,
X_3=F, A S or T,
X_4= S, A, Q, H, T, G or K,
X_5=N, T, Q, R, A, L, P or S,
X_6=Q, Y, A, L, F, M or S,
X_7=P, W, Q, or L,
X_8=M, L, I, G or V,
X_9=H, S or T,

b. a heavy chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6DX_7X_8X_9X_{10}X_{11}$SAAPVKG, wherein
X_1=R, A, or Q,
X_2=I, V, L, M, or Q,
X_3=R or K,
X_4=A, S, or G,
X_5=Q, K, E, S, V, I, H, L, R, or Y,
X_6=A, Q, R, L, M,
X_7=G, N, E, L,
X_8=G, A, D, E, Q,
X_9=T, E, Q, R, V, W,
X_{10}=T, Q, V, I, P, and
X_{11}=D, Q, V, F, W;

c. a heavy chain variable region CDR3 sequence comprises a sequence that differs by no more than one or two amino acid from the sequence VDRRNYYGMDV (SEQ ID NO: 5;

d. a light chain variable region CDR1 sequence comprises a sequence that differs by no more than one or two amino acids from the sequence RASQGIRNDLG (SEQ ID NO: 12);

e. a light chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6$S, wherein
X_1=A or N,
X_2=A, G, S, T, or V,
X_3=S, W, D, G, N, Q, H, or T,
X_4=R, Q, T, S, H, or K,
X_5=R, L, P, W, H, K, M, N, Q, S, T, or V, and
X_6=D, M, S, or V; and

f. a light chain variable region CDR3 sequence comprising a sequence $X_1QX_2X_3X_4X_5PWX_6$, wherein
X_1=L or V,

X_2=Q, M, or L,
X_3=R, K, Y, F, N, A, H, T, or W,
X_4=R, K, A, N, Q, L, M, S, or T,
X_5=M, T, V, A, I, Q, Y, L, P, K, R, or S, and
X_6=T, R, M, V, A, K, or S.

2. The antibody, or antigen binding fragment thereof, according to claim 1, wherein the antibody, or antigen binding fragment thereof, comprises:

a. a heavy chain variable region CDR1 sequence comprising a sequence $GX_1X_2X_3X_4X_5X_6DX_7X_8X_9$, wherein

b. X_1=F, D or E,
X_2=T, W or G,
X_3=F, A S or T,
X_4= S, Q, H, T, or K,
X_5=N, T, L or S,
X_6= Q, Y, A, L, or M,
X_7=P, W, or L,
X_8=M, L, or I
X_9=H, or S;

c. the heavy chain variable region CDR2 sequence comprising a sequence $X_1IX_2AX_3X_4DGGTTDSAAPVKG$, wherein
X_1=R or Q,
X_2=R or K,
X_3=Q, S, V, and
X_4=R, Q, A;

d. a heavy chain variable region CDR3 sequence comprises a sequence that differs by no more than one or two amino acid from the sequence VDRRNYYGMDV (SEQ ID NO: 5);

e. a light chain variable region CDR1 sequence comprises a sequence that differs by no more than one or two amino acids from the sequence RASQGIRNDLG (SEQ ID NO: 12);

f. the light chain variable region CDR2 sequence comprising a sequence $X_1X_2X_3X_4X_5X_6S$, wherein
X_1=A or N,
X_2=G, A, or S,
X_3=W, S, or G,
X_4=Q, T, or R,
X_5=R, L, or P, and
X_6=D, M, S, or V; and

g. the light chain variable region CDR3 sequence comprising a sequence $LQX_1X_2X_3X_4PWX_5$, wherein
X_1=Q, M, L,
X_2=R, Y, K, F, H, N,
X_3=R, K, N, A,

X_4=M, T, V, I, P, and
X_5=T, M, V.

3. The antibody, or antigen binding fragment thereof, according to claim 1, wherein:

   a. the heavy chain variable region CDR1 sequence comprises a sequence that differs by no more than one amino acid from the sequence NAWMS (SEQ ID NO: 3), SQWMS (SEQ ID NO: 39), or TYPMH (SEQ ID NO: 129);

   b. the heavy chain variable region CDR2 sequence comprises a sequence that differs by no more than one or two amino acids from the sequence RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4) or RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58);

   c. the heavy chain variable region CDR3 sequence comprises a sequence that differs by no more than one or two amino acid from the sequence VDRRNYYGMDV (SEQ ID NO: 5);

   d. the light chain variable region CDR1 sequence comprises a sequence that differs by no more than one or two amino acids from the sequence RASQGIRNDLG (SEQ ID NO: 12);

   e. the light chain variable region CDR2 sequence comprises a sequence that differs by no more than one amino acid from the sequence AASSLQS (SEQ ID NO: 13) or AGWQRDS (SEQ ID NO: 85); and

   f. the light chain variable region CDR3 sequence comprises a sequence that differs by no more than one amino acid from the sequence LQQNSYPWT (SEQ ID NO: 14), LQQRRMPWT (SEQ ID NO: 122), LQQYRVPWT (SEQ ID NO: 50), or LQMRRTPWT (SEQ ID NO: 104).

4. The antibody, or antigen binding fragment thereof, according to any one of claims 1-3, wherein heavy chain variable region CDR1 comprises NAWMS (SEQ ID NO: 3), SQWMS (SEQ ID NO: 39), or TYPMH (SEQ ID NO: 129).

5. The antibody, or antigen binding fragment thereof, according to any one of claims 1-4, wherein heavy chain variable region CDR2 comprises RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4) or RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58).

6. The antibody, or antigen binding fragment thereof, according to any one of claims 1-5, wherein heavy chain variable region CDR3 comprises VDRRNYYGMDV (SEQ ID NO: 5).

7. The antibody, or antigen binding fragment thereof, according to any one of claims 1-6, wherein light chain variable region CDR1 comprises RASQGIRNDLG (SEQ ID NO: 12),

8. The antibody, or antigen binding fragment thereof, according to any one of claims 1-7, wherein light chain variable region CDR2 comprises AASSLQS (SEQ ID NO: 13) or AGWQRDS (SEQ ID NO: 85);

9. The antibody, or antigen binding fragment thereof, according to any one of claims 1-8, wherein light chain variable region CDR3 comprises LQQNSYPWT (SEQ ID NO: 14), LQQRRMPWT (SEQ ID NO: 122), LQQYRVPWT (SEQ ID NO: 50), or LQMRRTPWT (SEQ ID NO: 104).

10. The antibody, or antigen binding fragment thereof, according to any one of claims 1-9, wherein

a. a heavy chain variable region CDR1 sequence comprises NAWMS (SEQ ID NO: 3);

b. a heavy chain variable region CDR2 sequence comprises RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4); and

c. a heavy chain variable region CDR3 sequence comprises VDRRNYYGMDV (SEQ ID NO: 5).

11. The antibody, or antigen binding fragment thereof, according to any one of claims 1-9, wherein

a. a heavy chain variable region CDR1 sequence comprises SQWMS (SEQ ID NO: 39);

b. a heavy chain variable region CDR2 sequence comprises RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4); and

c. a heavy chain variable region CDR3 sequence comprises VDRRNYYGMDV (SEQ ID NO: 5).

12. The antibody, or antigen binding fragment thereof, according to any one of claims 1-9, wherein

a. a heavy chain variable region CDR1 sequence comprises NAWMS (SEQ ID NO: 3);

b. a heavy chain variable region CDR2 sequence comprises RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58); and

c. a heavy chain variable region CDR3 sequence comprises VDRRNYYGMDV (SEQ ID NO: 5).

13. The antibody, or antigen binding fragment thereof, according to any one of claims 1-9, wherein

a. a heavy chain variable region CDR1 sequence comprises TYPMH (SEQ ID NO: 129);

b. a heavy chain variable region CDR2 sequence comprises RIRSKADGGTTDSAAPVKG (SEQ ID NO: 4); and

c. a heavy chain variable region CDR3 sequence comprises VDRRNYYGMDV (SEQ ID NO: 5).

14. The antibody, or antigen binding fragment thereof, according to any one of claims 1-13, wherein

a. a light chain variable region CDR1 sequence comprises RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprises AASSLQS (SEQ ID NO: 13); and

c. a light chain variable region CDR3 sequence comprises LQQNSYPWT (SEQ ID NO: 14).

15. The antibody, or antigen binding fragment thereof, according to any one of claims 1-13, wherein

a. a light chain variable region CDR1 sequence comprises RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprises AASSLQS (SEQ ID NO: 13) or AGWQRDS (SEQ ID NO: 85); and

c. a light chain variable region CDR3 sequence LQQYRVPWT (SEQ ID NO: 50).

16. The antibody, or antigen binding fragment thereof, according to any one of claims 1-13, wherein

a. a light chain variable region CDR1 sequence comprises RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprises AGWQRDS (SEQ ID NO: 85); and

c. a light chain variable region CDR3 sequence comprises LQQYRVPWT (SEQ ID NO: 50).

17. The antibody, or antigen binding fragment thereof, according to any one of claims 1-13, wherein

a. a light chain variable region CDR1 sequence comprises RASQGIRNDLG (SEQ ID NO: 12);

b. a light chain variable region CDR2 sequence comprises AGWQRDS (SEQ ID NO: 85); and

c. a light chain variable region CDR3 sequence comprises LQMRRTPWT (SEQ ID NO: 104).

18. The antibody, or antigen binding fragment thereof, according to any one of claims 1-13, wherein

a. a. a light chain variable region CDR1 sequence comprises RASQGIRNDLG (SEQ ID NO: 12);

b. b. a light chain variable region CDR2 sequence comprises AASSLQS (SEQ ID NO: 13); and

c. c. a light chain variable region CDR3 sequence comprises LQQRRMPWT (SEQ ID NO: 122).

19. The antibody, or antigen binding fragment thereof, according to any one of claims 1-9, wherein

d. a. a heavy chain variable region CDR1 sequence comprises NAWMS (SEQ ID NO: 3);

e. b. a heavy chain variable region CDR2 sequence comprises RIRAQRDGGTTDSAAPVKG (SEQ ID NO: 58);

f. c. a heavy chain variable region CDR3 sequence comprises VDRRNYYGMDV (SEQ ID NO: 5).

g. d. a light chain variable region CDR1 sequence comprises RASQGIRNDLG (SEQ ID NO: 12);

h. e. a light chain variable region CDR2 sequence comprises AGWQRDS (SEQ ID NO: 85); and

i. f. a light chain variable region CDR3 sequence comprises LQQYRVPWT (SEQ ID NO: 50).

20. The antibody, or antigen binding fragment thereof, according to any one of claims 1-19, further comprising a heavy chain variable region FW1 sequence comprising EVQLVESGGGLVKPGGSLRLSCAASG$X_1X_2X_3X_4$ (SEQ ID NO: 145), wherein X_1, X_2, X_3, and X_4 are independently chosen from any amino acid.

21. The antibody, or antigen binding fragment thereof, according to claim 20, wherein X_1 is F or E.

22. The antibody, or antigen binding fragment thereof, according to any one of claims 20-21, wherein X_2 is T or W.

23. The antibody, or antigen binding fragment thereof, according to any one of claims 20-22, wherein X_3 is F or A.

24. The antibody, or antigen binding fragment thereof, according to claim 20-23, wherein X_4 is S or Q.

25. The antibody, or antigen binding fragment thereof, according to any one of claims 1-24, further comprising a heavy chain variable region FW2 sequence comprising WVRQAPGKGLEWIG (SEQ ID NO: 7).

26. The antibody, or antigen binding fragment thereof, according to any one of claims 1-25, further comprising a heavy chain variable region FW3 sequence comprising RFTISRDDSKNTLYLQMNSLKTEDTAVYYCTT (SEQ ID NO: 8).

27. The antibody, or antigen binding fragment thereof, according to any one of claims 1-26, further comprising a heavy chain variable region FW4 sequence comprising WGQGTTVTVSS (SEQ ID NO: 9).

28. The antibody, or antigen binding fragment thereof, according to any one of claims 1-27, further comprising a light chain variable region FW1 sequence comprising DIQMTQSPSSLSASVGDRVTITC (SEQ ID NO: 15).

29. The antibody, or antigen binding fragment thereof, according to any one of claims 1-28, further comprising a light chain variable region FW2 sequence comprising WYQQKPGKAPKRLIY (SEQ ID NO: 16).

30. The antibody, or antigen binding fragment thereof, according to any one of claims 1-29, further comprising a light chain variable region FW3 sequence comprising GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC (SEQ ID NO: 17).

31. The antibody, or antigen binding fragment thereof, according to any one of claims 1-30, further comprising a light chain variable region FW4 sequence comprising FGQGTKVEIK (SEQ ID NO: 18).

32. The antibody, or antigen binding fragment thereof, according to any one of claims 1-31, wherein the antibody, or antigen binding fragment thereof, has an Fc domain from an IgG1, IgG2, IgG3, or IgG4.

33. The antibody, or antigen binding fragment thereof, according to claim 32, wherein the Fc domain, comprises at least one mutation capable of reducing the ADCC activity of the anti-5T4 antibody.

34. The antibody, or antigen binding fragment thereof, according to claim 32 or 33, wherein the Fc domain is modified to have improved half-life.

35. The antibody, or antigen binding fragment thereof, according to claim 34, wherein the Fc domain is mutated to include one, two, or all three of the following substitutions M252Y, S254T, and T256E, wherein the numbering corresponds to the EU index in Kabat.

36. The antibody, or antigen binding fragment thereof, according to any one according to claims 32 to 35, wherein the Fc domain comprises at least one amino acid modification introducing a derivatizable functional group.

37. The antibody, or antigen binding fragment thereof, according to claim 36 wherein the derivatizable functional group is a sulfhydryl side chain of a cysteine amino acid.

38. The antibody, or antigen binding fragment thereof, according to claim 37, wherein the antibody, or antigen binding fragment thereof, has an Fc domain having a cysteine substitution at one or more of the following positions 239, 248, 254, 273, 279, 282, 284, 286, 287, 289, 297, 298, 312, 324, 326, 330, 335, 337, 339, 350, 355, 356, 359, 360, 361, 375, 383, 384, 389, 398, 400, 413, 415, 418, 422, 440, 441, 442, 443 and 446, wherein the numbering corresponds to the EU index in Kabat.

39. The antibody, or antigen binding fragment thereof, according to claim 37 or 38, wherein the antibody, or antigen binding fragment thereof, has an Fc domain having a cysteine insertion between positions 239 and 240, wherein the numbering corresponds to the EU index in Kabat.

40. The antibody, or antigen binding fragment thereof, according to any one according to claims 32 to 39, wherein the antibody, or antigen binding fragment thereof, has an Fc domain having an F at position 234 and a C at position 239, wherein the numbering corresponds to the EU index in Kabat.

41. The antibody, or antigen binding fragment thereof, according to any one of claims 32 to 40, wherein the antibody, or antigen binding fragment thereof, has an Fc domain having an F at position 234, a C at position 239, and a C at position 442, wherein the numbering corresponds to the EU index in Kabat.

42. The antibody, or antigen binding fragment thereof, according to any one of claims 32 to 41, wherein the antibody, or antigen binding fragment thereof, has an Fc domain having a C inserted between position 239 and 240, wherein the numbering corresponds to the EU index in Kabat.

43. The antibody, or antigen binding fragment thereof, according to any one of claims 32 to 42, wherein the antibody, or antigen binding fragment thereof, has an Fc domain having a C at position 442, wherein the numbering corresponds to the EU index in Kabat.

44. The antibody, or antigen binding fragment thereof, according to any one of claims 32 to 43, wherein the antibody, or antigen binding fragment thereof, has an Fc domain having a C at position 442 and a C inserted between positions 239 and 240, wherein the numbering corresponds to the EU index in Kabat.

45. The antibody, or antigen binding fragment thereof, according to any one of claims 1 to 44, wherein the antibody, or antigen binding fragment thereof, is fully human, humanized antibody, or chimeric.

46. The antigen binding fragment thereof according to any one of claims 1 to 45, wherein the antigen binding fragment thereof is a single chain antibody, a single chain variable fragment (scFv), a Fab fragment, or a $F(ab')_2$ fragment.

47. The antibody, or antigen binding fragment thereof, according to any one of claims 1-46, wherein the antibody, or antigen binding fragment thereof, has an IC50:

(i) of 325nM or less;

(ii) of 100nM or less;

(iii) of 20nM or less;

(iv) of 10nM or less;

(v) of 5nM or less; or

(vi) of 1nM or less;

in a competitive binding assay to 5T4 antigen.

48. An antibody-drug conjugate comprising:

(i) the antibody, or antigen binding fragment thereof, according to any one of claims 1-47;

(ii) a chemotherapeutic agent; and

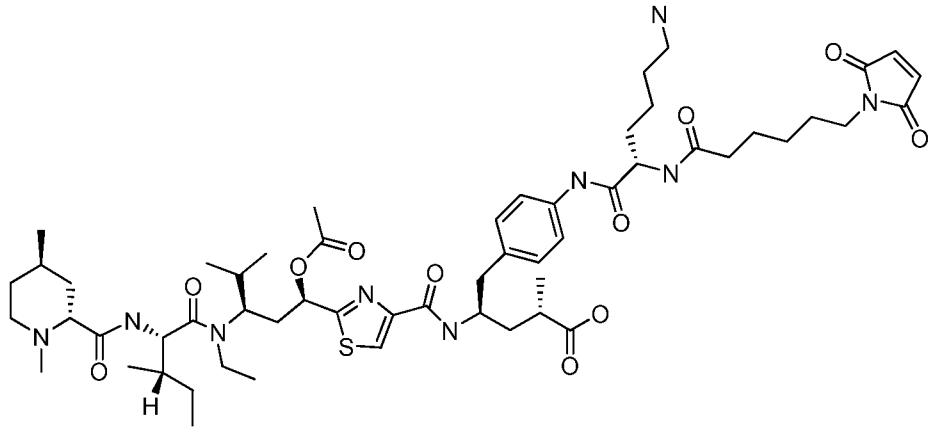
(iii) optionally a linker.

49. The antibody-drug conjugate according to claim 48, wherein the cytotoxic agent is a cancer chemotherapeutic agent.

50. The antibody-drug conjugate according to any one of claims 48 to 49, wherein chemotherapeutic agent is selected from the group consisting of amanitin, auristatin, daunomycin, doxorubicin, duocarmycin, dolastatin, enediyne, lexitropsin, taxane, puromycin, maytansinoid, vinca alkaloid, tubulysin , pyrrolobenzodiazepine, AFP, MMAF, MMAE, AEB, AEVB, auristatin E, paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretatstatin, chalicheamicin, maytansine, DM-1, vinblastine, methotrexate, and netropsin.

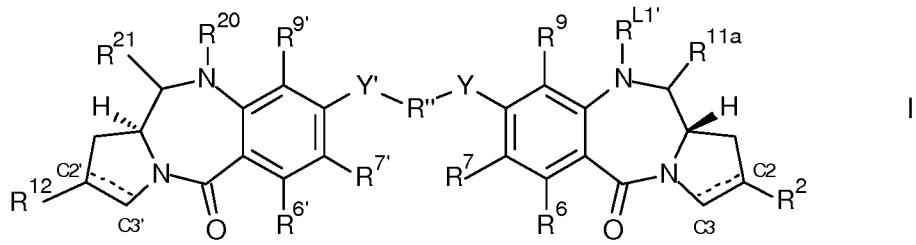
51. The antibody-drug conjugate according to any one of claims 48 to 50, wherein the chemotherapeutic agent is a tubulysin or a derivative thereof.

52. The antibody-drug conjugate according to claim 51, wherein the tubulysin has the following structure:

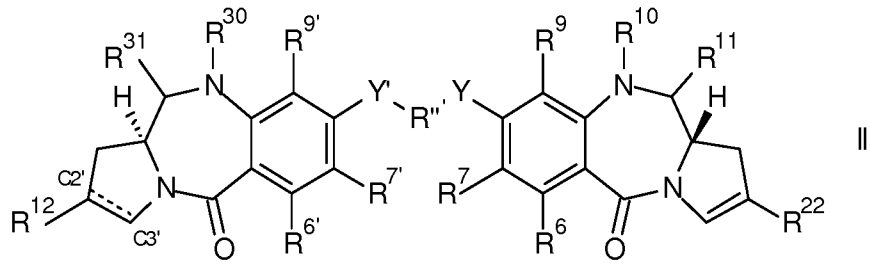


53. The antibody-drug conjugate according to any one of claims 48 to 50, wherein the chemotherapeutic agent is a pyrrolobenzodiazepine or a derivative thereof

54. The antibody-drug conjugate according to any one of claims 48-50 and 53 wherein the antibody drug conjugate is of formula L - $(D^L)_p$, where D^L is of formula **I** or **II**:



I



II

wherein:

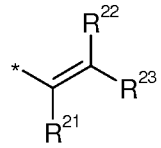
L is an antibody (Ab)or antigen binding fragment according to any one of claims 1-47;

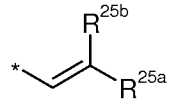
when there is a double bond present between C2' and C3', R^{12} is selected from the group consisting of:

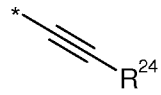
(ia) C_{5-10} aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C_{1-7} alkyl, C_{3-7} heterocyclyl and bis-oxy-C_{1-3} alkylene;

(ib) C_{1-5} saturated aliphatic alkyl;

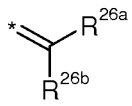
(ic) C_{3-6} saturated cycloalkyl;

(id)  ,wherein each of R21, R22 and R23 are independently selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl, where the total number of carbon atoms in the R12 group is no more than 5;

(ie)  , wherein one of R25a and R25b is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and

(if)  , where R24 is selected from: H; C1-3 saturated alkyl; C2-3 alkenyl; C2-3 alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

when there is a single bond present between C2' and C3',

R^{12} is  , where R^{26a} and R^{26b} are independently selected from H, F, C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C_{1-4} alkyl amido and C_{1-4} alkyl ester; or, when one of R^{26a} and R^{26b} is H, the other is selected from nitrile and a C_{1-4} alkyl ester;

R^6 and R^9 are independently selected from H, R, OH, OR, SH, SR, NH_2, NHR, NRR', nitro, Me_3Sn and halo;

where R and R' are independently selected from optionally substituted C_{1-12} alkyl, C_{3-20} heterocyclyl and C_{5-20} aryl groups;

R^7 is selected from H, R, OH, OR, SH, SR, NH_2, NHR, NHRR', nitro, Me_3Sn and halo;

R″ is a C_{3-12} alkylene group, which chain may be interrupted by one or more heteroatoms, e.g. O, S, NR^{N2} (where R^{N2} is H or C_{1-4} alkyl), and/or aromatic rings, e.g. benzene or pyridine;

Y and Y' are selected from O, S, or NH;

$R^{6'}$, $R^{7'}$, $R^{9'}$ are selected from the same groups as R^6, R^7 and R^9 respectively;

$R^{L1'}$ is a linker for connection to the antibody (Ab);

R^{11a} is selected from OH, OR^A, where R^A is C_{1-4} alkyl, and SO_zM, where z is 2 or 3 and M is a monovalent pharmaceutically acceptable cation;

R^{20} and R^{21} either together form a double bond between the nitrogen and carbon atoms to which they are bound or;

R^{20} is selected from H and R^C, where R^C is a capping group;

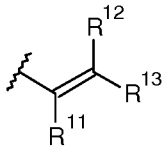
R^{21} is selected from OH, OR^A and SO_zM;

when there is a double bond present between C2 and C3, R^2 is selected from the group consisting of:

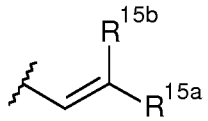
(ia) C_{5-10} aryl group, optionally substituted by one or more substituents selected from the group comprising: halo, nitro, cyano, ether, carboxy, ester, C_{1-7} alkyl, C_{3-7} heterocyclyl and bis-oxy-C_{1-3} alkylene;

(ib) C_{1-5} saturated aliphatic alkyl;

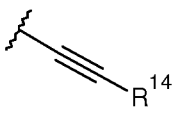
(ic) C3-6 saturated cycloalkyl;

(id) 

, wherein each of R11, R12 and R13 are independently selected from H, C1-3 saturated alkyl, C2-3 alkenyl, C2-3 alkynyl and cyclopropyl, where the total number of carbon atoms in the R2 group is no more than 5;

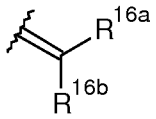
(ie) 

, wherein one of R15a and R15b is H and the other is selected from: phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl; and

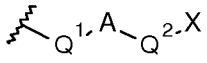
(if) 

, where R14 is selected from: H; C1-3 saturated alkyl; C2-3 alkenyl; C2-3 alkynyl; cyclopropyl; phenyl, which phenyl is optionally substituted by a group selected from halo, methyl, methoxy; pyridyl; and thiophenyl;

when there is a single bond present between C2 and C3,

R^2 is  , where R^{16a} and R^{16b} are independently selected from H, F, C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted by a group selected from C_{1-4} alkyl amido and C_{1-4} alkyl ester; or, when one of R^{16a} and R^{16b} is H, the other is selected from nitrile and a C_{1-4} alkyl ester;

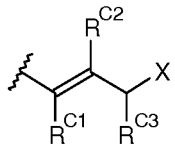
R^{22} is of formula IIIa, formula IIIb or formula IIIc:

(a)  IIIa

where A is a C_{5-7} aryl group, and either

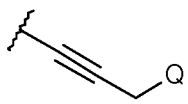
(i) Q^1 is a single bond, and Q^2 is selected from a single bond and -Z-$(CH_2)_n$-, where Z is selected from a single bond, O, S and NH and n is from 1 to 3; or

(ii) Q^1 is -CH=CH-, and Q^2 is a single bond;

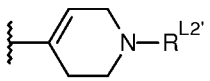
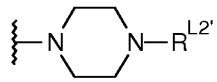
(b)  IIIb

where;

R^{C1}, R^{C2} and R^{C3} are independently selected from H and unsubstituted C_{1-2} alkyl;

(c)  IIIc

where Q is selected from O-RL2', S-RL2' and NRN-RL2', and RN is selected from H, methyl and ethyl

X is selected from the group comprising: O-$R^{L2'}$, S-$R^{L2'}$, CO_2-$R^{L2'}$, CO-$R^{L2'}$, NH-C(=O)-$R^{L2'}$, NHNH-$R^{L2'}$, CONHNH-$R^{L2'}$,  ,  , $NR^NR^{L2'}$, wherein R^N is selected from the group comprising H and C_{1-4} alkyl;

$R^{L2'}$ is a linker for connection to the antibody (Ab);

R^{10} and R^{11} either together form a double bond between the nitrogen and carbon atoms to which they are bound or;

R^{10} is H and R^{11} is selected from OH, OR^A and SO_zM;

R^{30} and R^{31} either together form a double bond between the nitrogen and carbon atoms to which they are bound or;

R^{30} is H and R^{31} is selected from OH, OR^A and SO_zM.

55. The conjugate according to claim 54, wherein R^7 is selected from H, OH and OR.

56. The conjugate according to claim 54, wherein R^7 is a C_{1-4} alkyloxy group.

57. The conjugate according to any one according to claims 54 to 56, wherein Y is O.

58. The conjugate according to any one of one according to claims 54 to 57, wherein R'' is C_{3-7} alkylene.

59. The conjugate according to any one according to claims 54 to58, wherein R^9 is H.

60. The conjugate according to any one according to claims 54 to 59, wherein R^6 is selected from H and halo.

61. The conjugate according to any one according to claims 54 to 60, wherein there is a double bond between C2' and C3', and R^{12} is a C_{5-7} aryl group.

62. The conjugate according to claim 61, wherein R^{12} is phenyl.

63. The conjugate according to any one according to claims 54 to 60, wherein there is a double bond between C2' and C3', and R^{12} is a C_{8-10} aryl group.

64. The conjugate according to any one according to claims 61 to 63, wherein R^{12} bears one to three substituent groups.

65. The conjugate according to any one according to claims 61 to 64, wherein the substituents are selected from methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl.

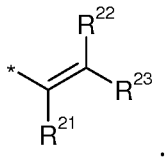
66. The conjugate according to any one according to claims 54 to 60, wherein there is a double bond between C2' and C3', and R^{12} is a C_{1-5} saturated aliphatic alkyl group.

67. A compound according to claim 66, wherein R^{12} is methyl, ethyl or propyl.

68. The conjugate according to any one according to claims 54 to 60, wherein there is a double bond between C2' and C3', and R^{12} is a C_{3-6} saturated cycloalkyl group.

69. The conjugate according to claim 68, wherein R^{12} is cyclopropyl.

70. The conjugate according to any one according to claims 54 to 60, wherein there is a double bond between C2' and C3', and R^{12} is a group of formula:



.

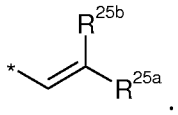
71. The conjugate according to claim 70, wherein the total number of carbon atoms in the R^{12} group is no more than 4.

72. The conjugate according to claim 71, wherein the total number of carbon atoms in the R^{12} group is no more than 3.

73. The conjugate according to any one according to claims 70 to 72, wherein one of R^{21}, R^{22} and R^{23} is H, with the other two groups being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

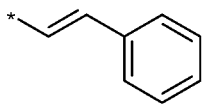
74. The conjugate according to any one according to claims 70 to 72, wherein two of R^{21}, R^{22} and R^{23} are H, with the other group being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

75. The conjugate according to any one according to claims 54 to 60, wherein there is a double bond between C2' and C3', and R^{12} is a group of formula:



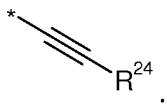
.

76. The conjugate according to claim 75, wherein R^{12} is the group:



.

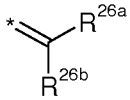
77. The conjugate according to any one according to claims 54 to 60, wherein there is a double bond between C2' and C3', and R^{12} is a group of formula:

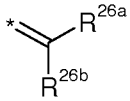


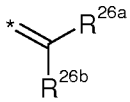
.

78. The conjugate according to claim 77, wherein R^{24} is selected from H, methyl, ethyl, ethenyl and ethynyl.

79. The conjugate according to claim 78, wherein R^{24} is selected from H and methyl.

80. The conjugate according to any one according to claims 54 to 60, wherein there is a single bond between C2' and C3', R^{12} is  and R^{26a} and R^{26b} are both H.

81. The conjugate according to any one according to claims 54 to 60, wherein there is a single bond between C2' and C3', R^{12} is  , and R^{26a} and R^{26b} are both methyl.

82. The conjugate according to any one according to claims 54 to 60, wherein there is a single bond between C2' and C3', R^{12} is  , one of R^{26a} and R^{26b} is H, and the other is selected from C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted.

83. The conjugate according to any one according to claims 54 to 82, wherein there is a double bond between C2 and C3, and R^2 is a C_{5-7} aryl group.

84. The conjugate according to claim 83, wherein R^2 is phenyl.

85. The conjugate according to any one according to claims 54 to 82, wherein there is a double bond between C2 and C3, and R^1 is a C_{8-10} aryl group.

86. A compound according to any one according to claims 83 to 85, wherein R^2 bears one to three substituent groups.

87. The conjugate according to any one according to claims 83 to 86, wherein the substituents are selected from methoxy, ethoxy, fluoro, chloro, cyano, bis-oxy-methylene, methyl-piperazinyl, morpholino and methyl-thiophenyl.

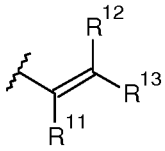
88. The conjugate according to any one according to claims 54 to 82, wherein there is a double bond between C2 and C3, and R^2 is a C_{1-5} saturated aliphatic alkyl group.

89. The conjugate according to claim 88, wherein R^2 is methyl, ethyl or propyl.

90. The conjugate according to any one according to claims 54 to 82, wherein there is a double bond between C2 and C3, and R^2 is a C_{3-6} saturated cycloalkyl group.

91. The conjugate according to claim 90, wherein R^2 is cyclopropyl.

92. The conjugate according to any one according to claims 54 to 82, wherein there is a double bond between C2 and C3, and R^2 is a group of formula:



.

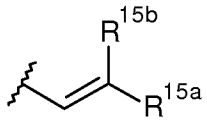
93. The conjugate according to claim 92, wherein the total number of carbon atoms in the R^2 group is no more than 4.

94. The conjugate according to claim 93, wherein the total number of carbon atoms in the R^2 group is no more than 3.

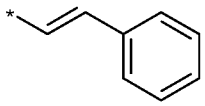
95. The conjugate according to any one according to claims 92 to 94, wherein one of R^{11}, R^{12} and R^{13} is H, with the other two groups being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

96. The conjugate according to any one according to claims 92 to 94, wherein two of R^{11}, R^{12} and R^{13} are H, with the other group being selected from H, C_{1-3} saturated alkyl, C_{2-3} alkenyl, C_{2-3} alkynyl and cyclopropyl.

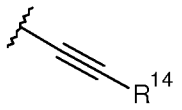
97. The conjugate according to any one according to claims 54 to 82, wherein there is a double bond between C2 and C3, and R^2 is a group of formula:



98. The conjugate according to claim 97, wherein R^2 is the group:

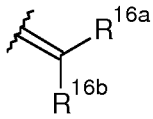


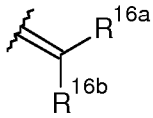
99. The conjugate according to any one according to claims 54 to 82, wherein there is a double bond between C2 and C3, and R^2 is a group of formula:



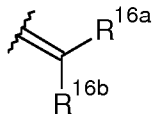
100. The conjugate according to claim 99, wherein R^{14} is selected from H, methyl, ethyl, ethenyl and ethynyl.

101. The conjugate according to claim 99, wherein R^{14} is selected from H and methyl.

102. The conjugate according to any one according to claims 54 to 82, wherein there is a single bond between C2 and C3, R^2 is  and R^{16a} and R^{16b} are both H.

103. The conjugate according to any one according to claims 54 to 82, wherein there is a single bond between C2 and C3, R^2 is  , and R^{16a} and R^{16b} are both methyl.

104. The conjugate according to any one according to claims 54 to 82, wherein



there is a single bond between C2 and C3, R^2 is , one of R^{16a} and R^{16b} is H, and the other is selected from C_{1-4} saturated alkyl, C_{2-3} alkenyl, which alkyl and alkenyl groups are optionally substituted.

105. The conjugate according to any one according to claims 54 to 104, wherein R^{11a} is OH.

106. The conjugate according to any one according to claims 54 to 105, wherein R^{21} is OH.

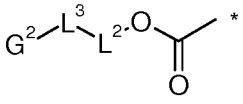
107. The conjugate according to any one according to claims 54 to 105, wherein R^{21} is OMe.

108. The conjugate according to any one according to claims 54 to 107, wherein R^{20} is H.

109. The conjugate according to any one according to claims 54 to 107, wherein R^{20} is R^C.

110. The conjugate according to claim 109, wherein R^C is selected from the group consisting of: Alloc, Fmoc, Boc, Troc, Teoc, Psec, Cbz and PNZ.

111. The conjugate according to claim 109, wherein R^C is a group:



where the asterisk indicates the point of attachment to the N10 position, G^2 is a terminating group, L^3 is a covalent bond or a cleavable linker L^1, L^2 is a covalent bond or together with OC(=O) forms a self-immolative linker.

112. The conjugate according to claim 111, wherein G^2 is Ac or Moc or is selected from the group consisting of: Alloc, Fmoc, Boc, Troc, Teoc, Psec, Cbz and PNZ.

113. The conjugate according to any one according to claims 54 to 105, wherein R^{20} and R^{21} together form a double bond between the nitrogen and carbon atoms to which they are bound.

114. The conjugate according to any one according to claims 54 to 82, wherein R^{22} is of formula IIIa, and A is phenyl.

115. The conjugate according to any one according to claims 54 to 82 and claim 114, wherein R^{22} is of formula IIa, and Q^1 is a single bond.

116. The conjugate according to claim 114, wherein Q^2 is a single bond.

117. The conjugate according to claim 114, wherein Q^2 is $-Z-(CH_2)_n-$, Z is O or S and n is 1 or 2.

118. The conjugate according any one according to claims 54 to 82and claim 114, wherein R^{22} is of formula IIIa, and Q^1 is -CH=CH-.

119. The conjugate according to any one according to claims 54 to 82, wherein R^{22} is of formula IIIb,

and R^{C1}, R^{C2} and R^{C3} are independently selected from H and methyl.

120. The conjugate according to claim 119, wherein R^{C1}, R^{C2} and R^{C3} are all H.

121. The conjugate according to claim 119, wherein R^{C1}, R^{C2} and R^{C3} are all methyl.

122. The conjugate according to any one according to claims 54 to 82 and claims 114 to 121, wherein R^{22} is of formula IIIa or formula IIIb and X is selected from O-$R^{L2'}$, S-$R^{L2'}$, CO_2-$R^{L2'}$, -N-C(=O)-$R^{L2'}$ and NH-$R^{L2'}$.

123. The conjugate according to claim 122, wherein X is NH-$R^{L2'}$.

124. The conjugate according to any one according to claims 54 to 82, wherein R^{22} is of formula IIIc, and Q is NR^N-$R^{L2'}$.

125. The conjugate according to claim 124, wherein R^N is H or methyl.

126. The conjugate according to any one according to claims 54 to 82, wherein R^{22} is of formula IIIc, and Q is O-$R^{L2'}$ or S-$R^{L2'}$.

127. The conjugate according to any one according to claims 54 to 82 and claims 114 to 126, wherein R^{11} is OH.

128. The conjugate according to any one according to claims 54 to 82 and claims 114 to 126, wherein R^{11} is OMe.

129. The conjugate according to any one according to claims 54 to 82 and claims 114 to 128, wherein R^{10} is H.

130. The conjugate according to any one according to claims 54 to 82 and claims 114 to 126, wherein R^{10} and R^{11} together form a double bond between the nitrogen and carbon atoms to which they are bound.

131. The conjugate according to any one according to claims 54 to 82 and claims 114 to 130, wherein R^{31} is OH.

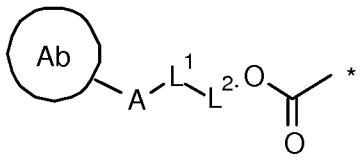
132. The conjugate according to any one according to claims 54 to 82 and claims 114 to 130, wherein R^{31} is OMe.

133. The conjugate according to any one according to claims 54 to 82 and claims 114 to 132, wherein R^{30} is H.

134. The conjugate according to any one according to claims 54 to 82 and claims 114 to 130, wherein R^{30} and R^{31} together form a double bond between the nitrogen and carbon atoms to which they are bound.

135. The conjugate according to any one according to claims 54 to 134, wherein $R^{6'}$, $R^{7'}$, $R^{9'}$, and Y' are the same as R^6, R^7, R^9, and Y.

136. The conjugate according to any one according to claims 54 to 135 wherein, wherein L-$R^{L1'}$ or L-$R^{L2'}$ is a group:



where the asterisk indicates the point of attachment to the PBD, Ab is the antibody, L^1 is a cleavable linker, A is a connecting group connecting L^1 to the antibody, L^2 is a covalent bond or together with -OC(=O)- forms a self-immolative linker.

137. The conjugate according to claim 136, wherein L^1 is enzyme cleavable.

138. The conjugate according to claim 136 or claim 84, wherein L^1 comprises a contiguous sequence of amino acids.

139. The conjugate according to claim 138, wherein L^1 comprises a dipeptide and the group -X_1-X_2- in dipeptide, -NH-X_1-X_2-CO-, is selected from:

-Phe-Lys-,

-Val-Ala-,

-Val-Lys-,

-Ala-Lys-,

-Val-Cit-,

-Phe-Cit-,

-Leu-Cit-,

-Ile-Cit-,

-Phe-Arg-,

-Trp-Cit-.

140. The conjugate according to claim 139, wherein the group -X_1-X_2- in dipeptide, -NH-X_1-X_2-CO-, is selected from:

-Phe-Lys-,

-Val-Ala-,

-Val-Lys-,

-Ala-Lys-,

-Val-Cit-.

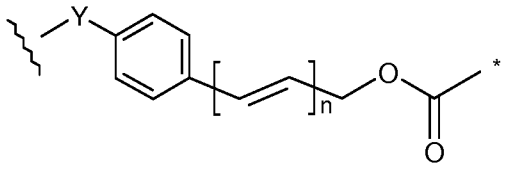
141. The conjugate according to claim 140, wherein the group -X_1-X_2- in dipeptide, -NH-X_1-X_2-CO-, is -Phe-Lys-, -Val-Ala- or -Val-Cit-.

142. The conjugate according to any one according to claims 139 to 141, wherein the group X_2-CO- is connected to L^2.

143. The conjugate according to any one according to claims 139 to 142, wherein the group NH-X_1- is connected to A.

144. The conjugate according to any one according to claims 139 to 143, wherein L^2 together with OC(=O) forms a self-immolative linker.

145. The conjugate according to claim 144, wherein C(=O)O and L^2 together form the group:

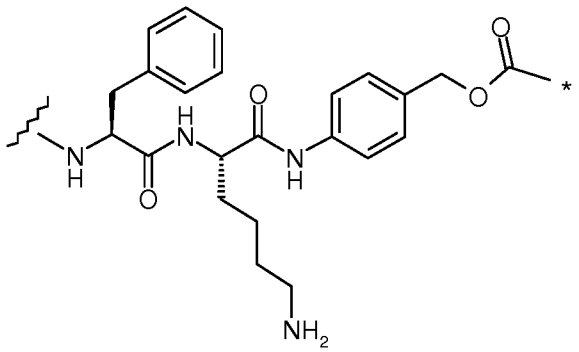


where the asterisk indicates the point of attachment to the PBD, the wavy line indicates the point of attachment to the linker L^1, Y is NH, O, C(=O)NH or C(=O)O, and n is 0 to 3.

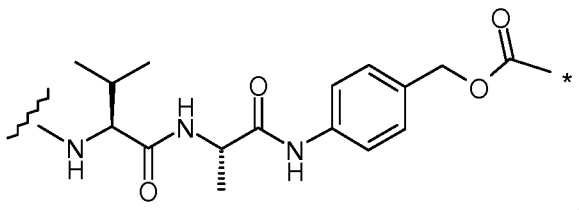
146. The conjugate according to claim 145, wherein Y is NH.

147. The conjugate according to claim 145 or claim 146, wherein n is 0.

148. The conjugate according to claim 146, wherein L^1 and L^2 together with -OC(=O)- comprise a group selected from:



or

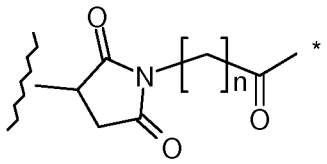


where the asterisk indicates the point of attachment to the PBD, and the wavy line indicates the point of attachment to the remaining portion of the linker L^1 or the point of attachment to A.

149. The conjugate according to claim 148, wherein the wavy line indicates the point of attachment to A.

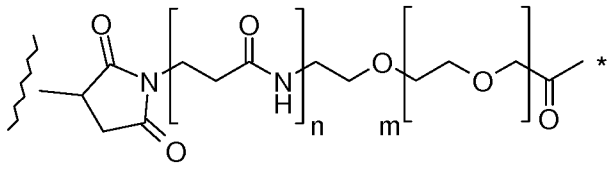
150. The conjugate according to any one according to claims 146 to 149, wherein A is:

(i)



where the asterisk indicates the point of attachment to L^1, the wavy line indicates the point of attachment to the antibody, and n is 0 to 6; or

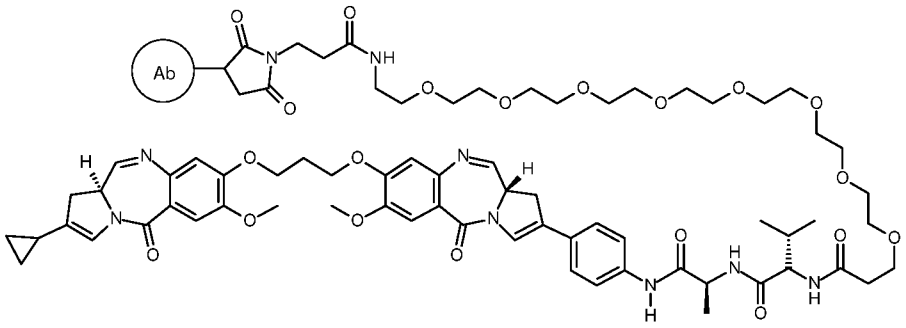
(ii)



where the asterisk indicates the point of attachment to L^1, the wavy line indicates the point of attachment to the antibody, n is 0 or 1, and m is 0 to 30.

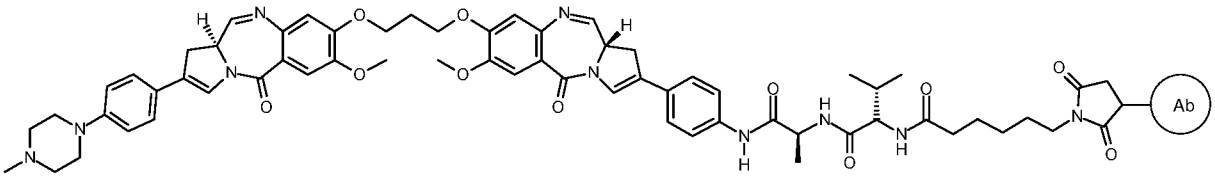
151. A conjugate according to according to any one of claims 54 to 150 wherein the conjugate is selected from the following formulae:

ConjA:



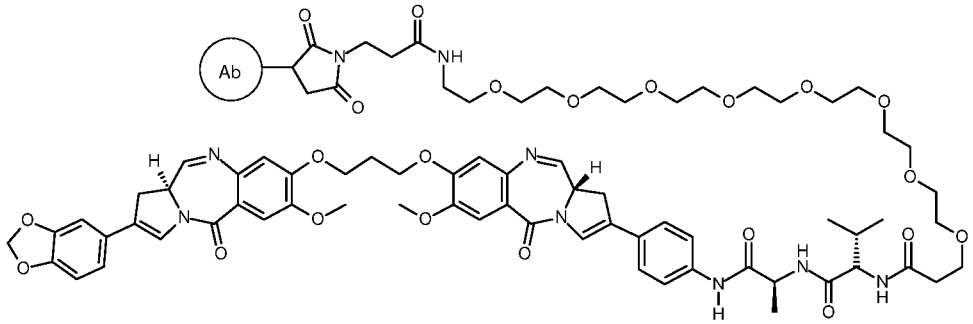
ConjA

ConjB:



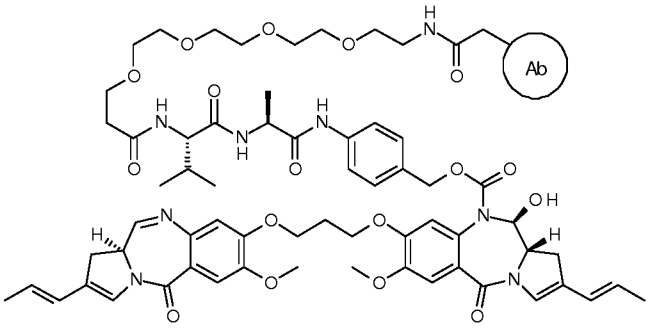
ConjB

ConjC:



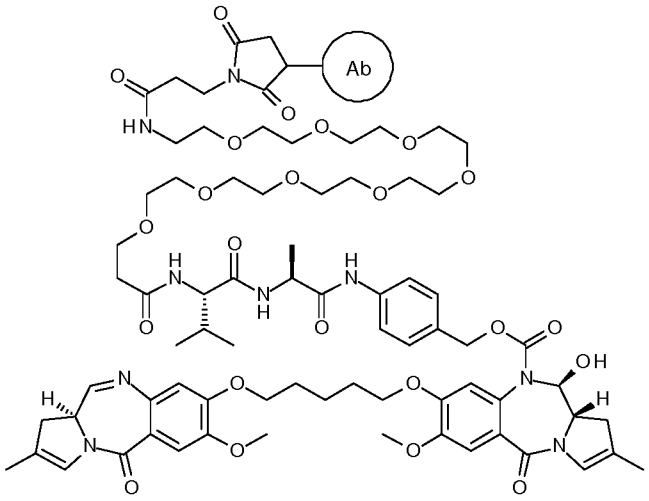
ConjC

ConjD:



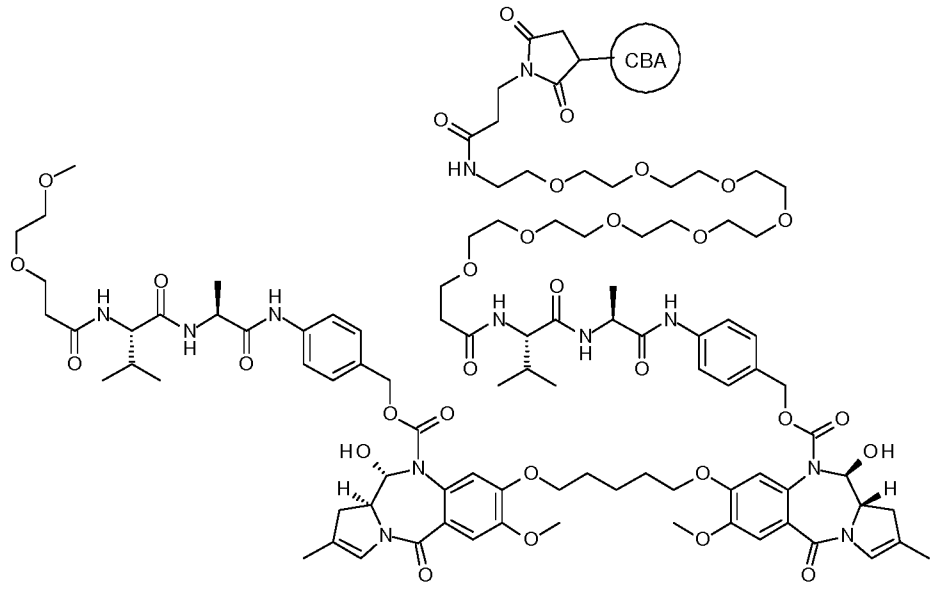
ConjD

ConjE:



ConjE

or ConjF



ConjF

152. The antibody-drug conjugate according to any one of claims 48 to 151, wherein the cytotoxic agent is conjugated to the antibody, or antigen binding fragment thereof, through at least one derivatizable group.

153. The antibody-drug conjugate according to claim 152 wherein the derivatizable group comprises at least one cysteine residue.

154. The antibody-drug conjugate according to any one of claims 48 to 153, wherein the cytotoxic agent is conjugated to the antibody, or antigen binding fragment thereof, through a thiol-maleimide linkage.

155. A antibody-imaging agent conjugate comprising:

(i) the antibody, or antigen binding fragment thereof, according to any one according to claims 1-47;

(ii) an imaging agent; and

(iii) optionally a linker.

156. The antibody-imaging agent conjugate according to claim 57, wherein the imaging agent comprises a flourophore, a PET label, a SPECT label, a MRI label, a radioisotope, an enzyme, a luminescent label, a bioluminescent label, a magnetic label, biotin, a contrast agent, a quantum dot, or a nanoparticle.

157. A synthetic or recombinant nucleic acid sequence encoding the antibody, or antigen binding fragment thereof, according to any one of claims 1-47.

158. The nucleic acid sequence according to claim 157, wherein the nucleic acid comprises a sequence chosen from sequences encoding the heavy chain: SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127.

159. The nucleic acid sequence according to claim 157, wherein the nucleic acid encoding the heavy chain comprises a sequence chosen from one that is 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127.

160. The nucleic acid sequence according to any one of claims 157-159, wherein the nucleic acid sequence comprises a sequence encoding heavy chain CDR1, CDR2, and CDR3 wherein the nucleic acids encode CDR residues identical to the CDR residues encoded by SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127.

161. The nucleic acid according to claim 157, wherein the nucleic acid sequence comprises a sequence encoding heavy chain CDR1, CDR2, and CDR3 wherein the nucleic acids encoding CDR residues result in no more than one or two amino acid differences in each CDR compared to the CDR residues encoded by SEQ ID NO: 1, 19, 37, 55, 73, 91, 109, or 127.

162. The nucleic acid according to claim 157, wherein the nucleic acid comprises a sequence chosen from sequences encoding the light chain: SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136.

163. The nucleic acid according to claim 157, wherein the nucleic acid encoding the light chain comprises a sequence chosen from one that is 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136.

164. The nucleic acid sequence according to any one of claims 157 to 163, wherein the nucleic acid sequence comprises a sequence encoding light chain CDR1,

CDR2, and CDR3 wherein the nucleic acids encoding CDR residues are identical to the CDR residues encoded by SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136.

165. The nucleic acid according to claim 157, wherein the nucleic acid sequence comprises a sequence encoding light chain CDR1, CDR2, and CDR3 wherein the nucleic acids encoding CDR residues result in no more than one or two amino acid differences in each CDR compared to the CDR residues encoded by SEQ ID NO: 10, 28, 46, 64, 82, 100, 118, or 136.

166. A vector comprising a nucleic acid according to any one of claims to 157 to 165.

167. A host cell containing the vector of claim 166.

168. An isolated antibody producing cell capable of producing the antibody, or antigen binding fragment thereof, according to any one of claims 1-45.

169. A method of inhibiting growth of a cancer cell comprising exposing a cancer cell to the antibody-drug conjugate according to any one of claims 48 to 154.

170. A method of treating cancer in a subject comprising administering the antibody-drug conjugate according to any one of claims 48 to 154 to the subject.

171. The method according to claim 170, wherein the subject is a human patient.

172. A method of detecting a cancer cell expressing the 5T4 cell surface antigen comprising exposing a cell to the antibody-imaging agent conjugate according to any one of claims 155 to 156 and determining whether the antibody-imaging agent binds to the cell.

173. A method of diagnosing cancer expressing the 5T4 cell surface antigen comprising administering the antibody-imaging agent conjugate according to any one of claims 155 to156 to the patient and detecting the imaging agent.

174. The method according to any one of claims 167 to 173, wherein the cancer is melanoma, lung cancer, colorectal cancer, gastric cancer, renal cancer, ovarian cancer, and leukemia

175. The method according to any one of claims 167 to 173, wherein the cancer is melanoma, small cell lung cancer, non-small cell lung cancer, glioma, hepatocellular carcinoma, thyroid tumor, gastric cancer, prostate cancer, breast cancer, ovarian cancer, bladder cancer, lung cancer, glioblastoma, endometrial cancer, kidney cancer, colon cancer, pancreatic cancer, esophageal carcinoma, head and neck cancers, mesothelioma, sarcomas, biliary cancer, small bowel adenocarcinoma, pediatric malignancies and epidermoid carcinoma.

FIG. 1

5T4-A07_GL
EVQLVESGGGLVKPGGSLRLSCAASGFTFS**NAWMS**----WVRQAPGKGLEWIG**RIRSKA**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0060
EVQLVESGGGLVKPGGSLRLSCAASG**EWA**S**SQWMS**----WVRQAPGKGLEWIG**RIRSKA**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0065
EVQLVESGGGLVKPGGSLRLSCAASGFTFS**NAWMS**----WVRQAPGKGLEWIG**RIRAQR**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0068
EVQLVESGGGLVKPGGSLRLSCAASGFTFS**NAWMS**----WVRQAPGKGLEWIG**RIRSKA**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0069
EVQLVESGGGLVKPGGSLRLSCAASGFTFS**NAWMS**----WVRQAPGKGLEWIG**RIRSKA**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0107
EVQLVESGGGLVKPGGSLRLSCAASG**EWA**S**SQWMS**----WVRQAPGKGLEWIG**RIRSKA**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0108
EVQLVESGGGLVKPGGSLRLSCAASGFTFS**NAWMS**----WVRQAPGKGLEWIG**RIRAQR**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0112
EVQLVESGGGLVKPGGSLRLSCAASGFTFS**NAWMS**----WVRQAPGKGLEWIG**RIRASQ**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

5t4_0113
EVQLVESGGGLVKPGGSLRLSCAASGFTFQTYPMH----WVRQAPGKGLEWIG**RIRSKA**---**DGGTTDSAAPVKG**
RFTISRDDSKNTLYLQMNSL--KTEDTAVYYCTTV**DRRNYYGM**----------**DV**WGQGTTVTVSS

FIG. 2


5T4-A07_GL
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAA-----SSLQSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQNSYP---------WTFGQGTKVEI---K
5t4_0065
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAA-----SSLQSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQNSYP---------WTFGQGTKVEI---K
5t4_0112
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAA-----SSLQSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQRRMP---------WTFGQGTKVEI---K
5t4_0068
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAG-----WQRDSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQYRVP---------WTFGQGTKVEI---K
5t4_0060
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAA-----SSLQSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQYRVP---------WTFGQGTKVEI---K
5t4_0113
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAA-----SSLQSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQRRMP---------WTFGQGTKVEI---K
5t4_0069
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAG-----WQRDSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQMRRTP---------WTFGQGTKVEI---K
5t4_0108
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAG-----WQRDSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQYRVP---------WTFGQGTKVEI---K
5t4_0107
DIQMTQSPSSLSASVGDRVTITCRASQ--------GIRNDLGWYQQKPGKAPKRLIYAG-----WQRDSGVPSRFSGSGSG--
TEFTLTISSLQPEDFATYYCLQQYRVP---------WTFGQGTKVEI---K

FIG. 3

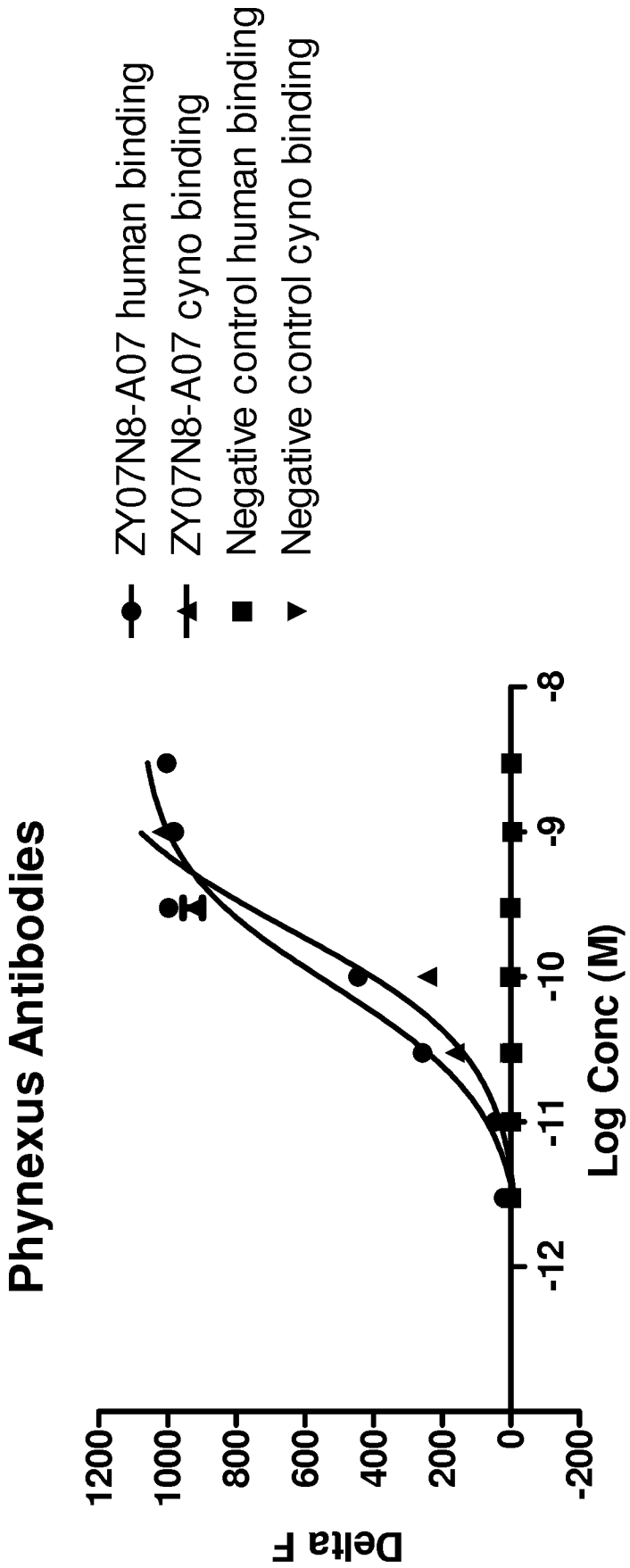

Phynexus Antibodies
ZY07N8-A07 human binding
ZY07N8-A07 cyno binding
Negative control human binding
Negative control cyno binding
Delta F
1200
1000
800
600
400
200
0
-200
-12
-11
-10
-9
-8
Log Conc (M)

FIG. 4

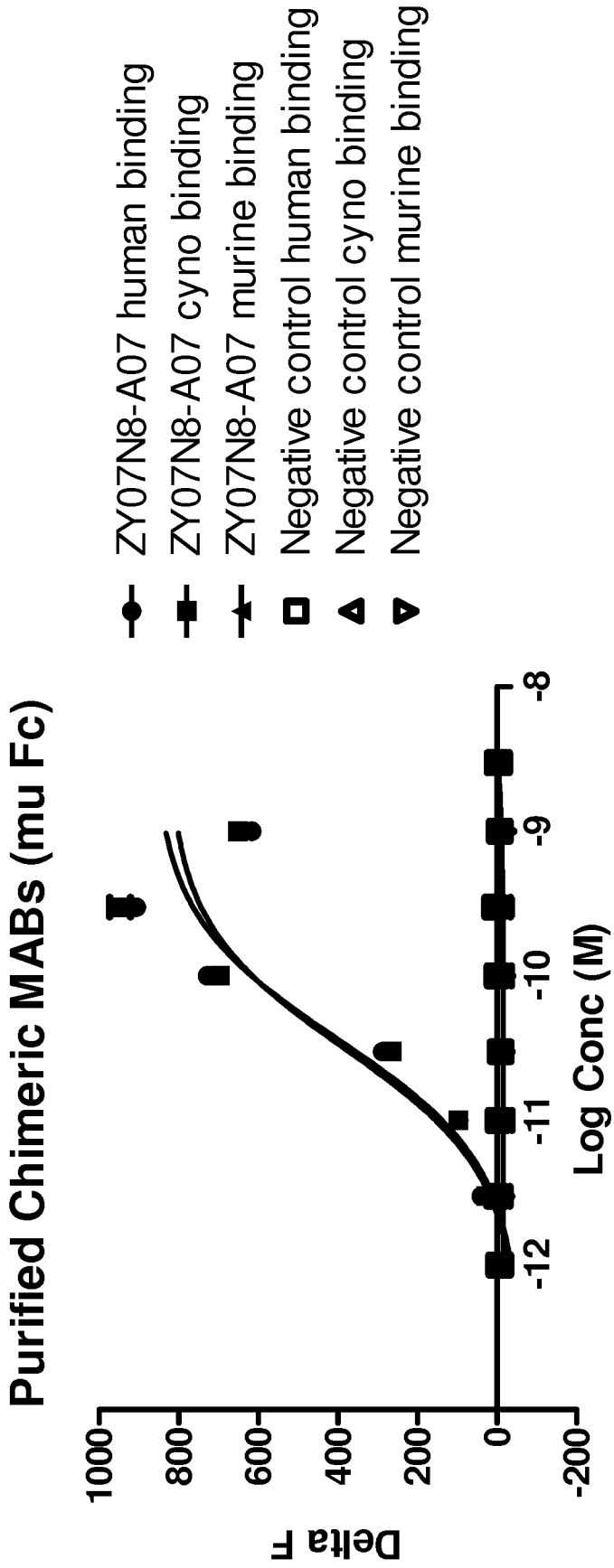

Purified Chimeric MABs (mu Fc)
ZY07N8-A07 human binding
ZY07N8-A07 cyno binding
ZY07N8-A07 murine binding
Negative control human binding
Negative control cyno binding
Negative control murine binding
Delta F
1000
800
600
400
200
0
-200
-12
-11
-10
-9
-8
Log Conc (M)

FIG. 5

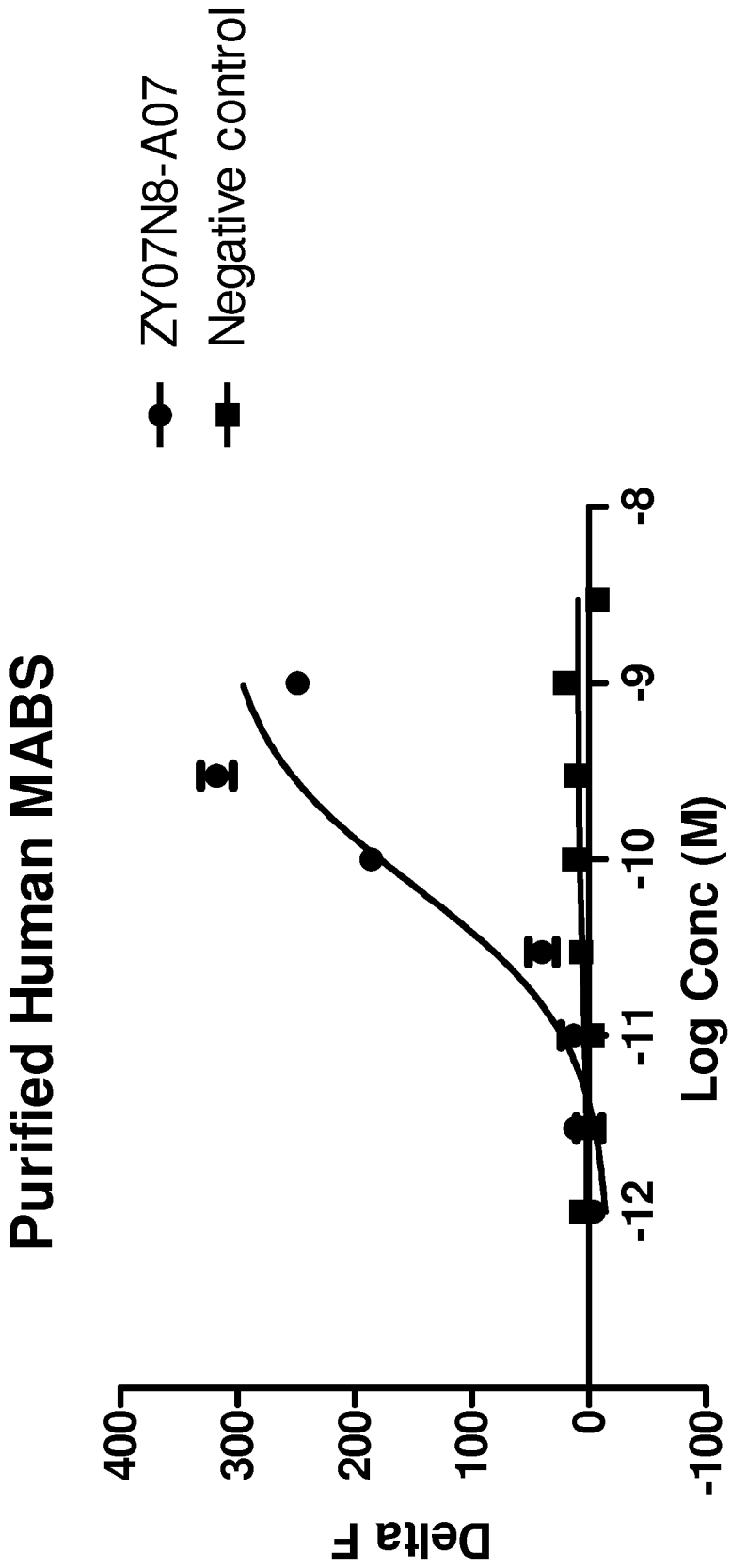

Purified Human MABS
ZY07N8-A07
Negative control
Delta F
400
300
200
100
0
-100
-12
-11
-10
-9
-8
Log Conc (M)

FIG. 6

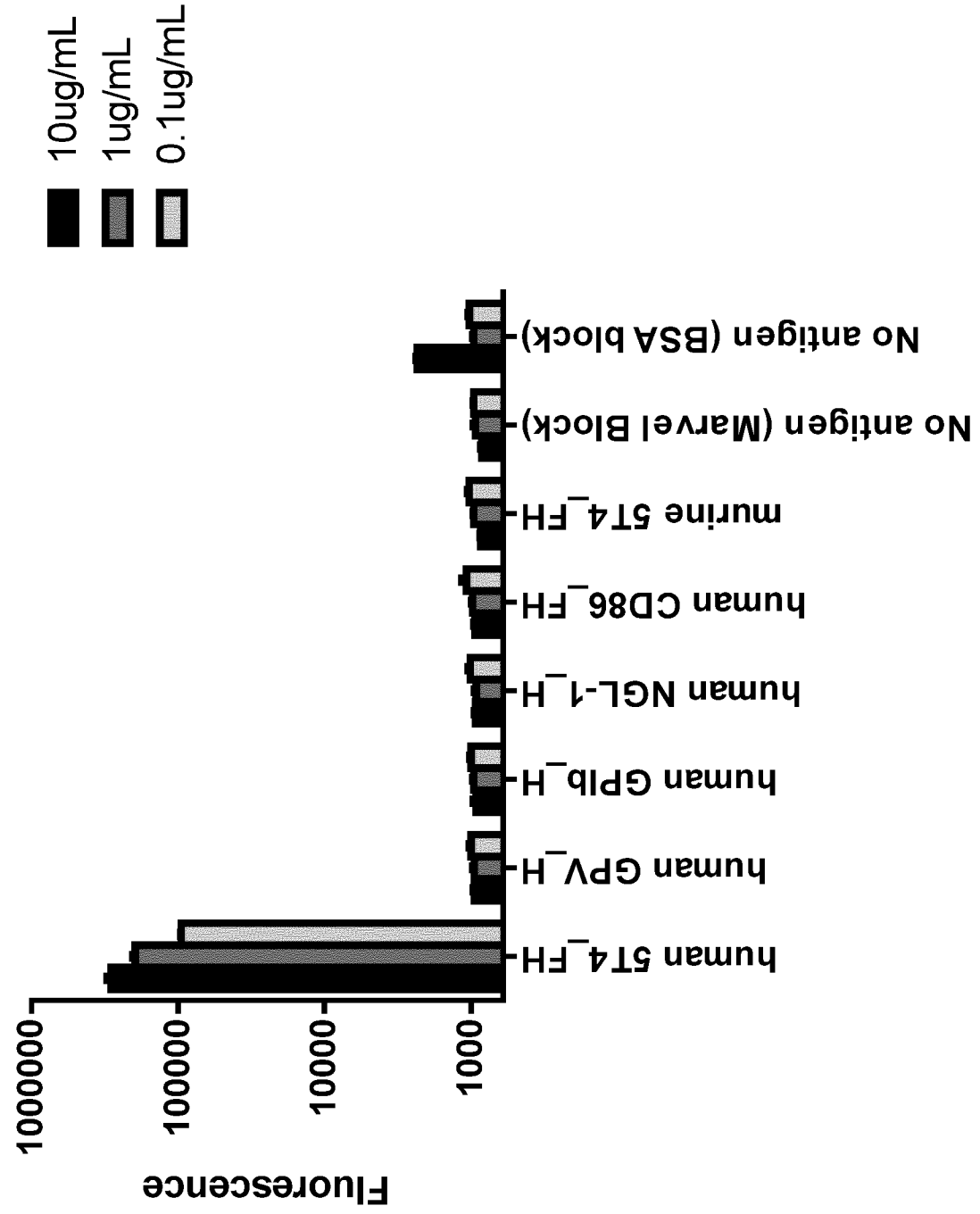

ZY07N8-A07
10ug/mL
1ug/mL
0.1ug/mL
Fluorescence
1000000
100000
10000
1000
human 5T4_FH
human GPV_H
human GPIb_H
human NGL-1_H
human CD86_FH
murine 5T4_FH
No antigen (Marvel Block)
No antigen (BSA block)

FIG. 7

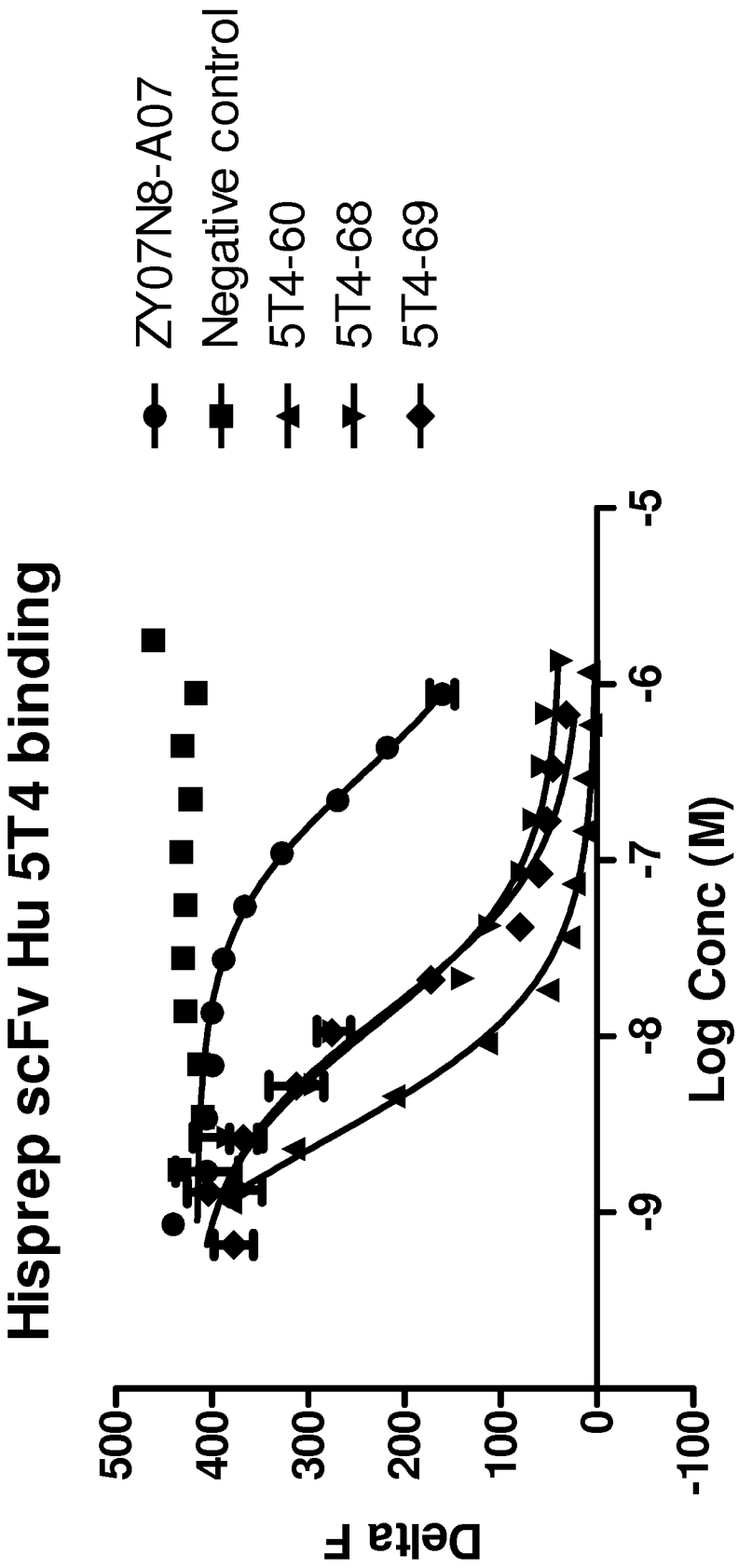

Hisprep scFv Hu 5T4 binding
ZY07N8-A07
Negative control
5T4-60
5T4-68
5T4-69
Delta F
500
400
300
200
100
0
-100
Log Conc (M)
-5
-6
-7
-8
-9

FIG. 8

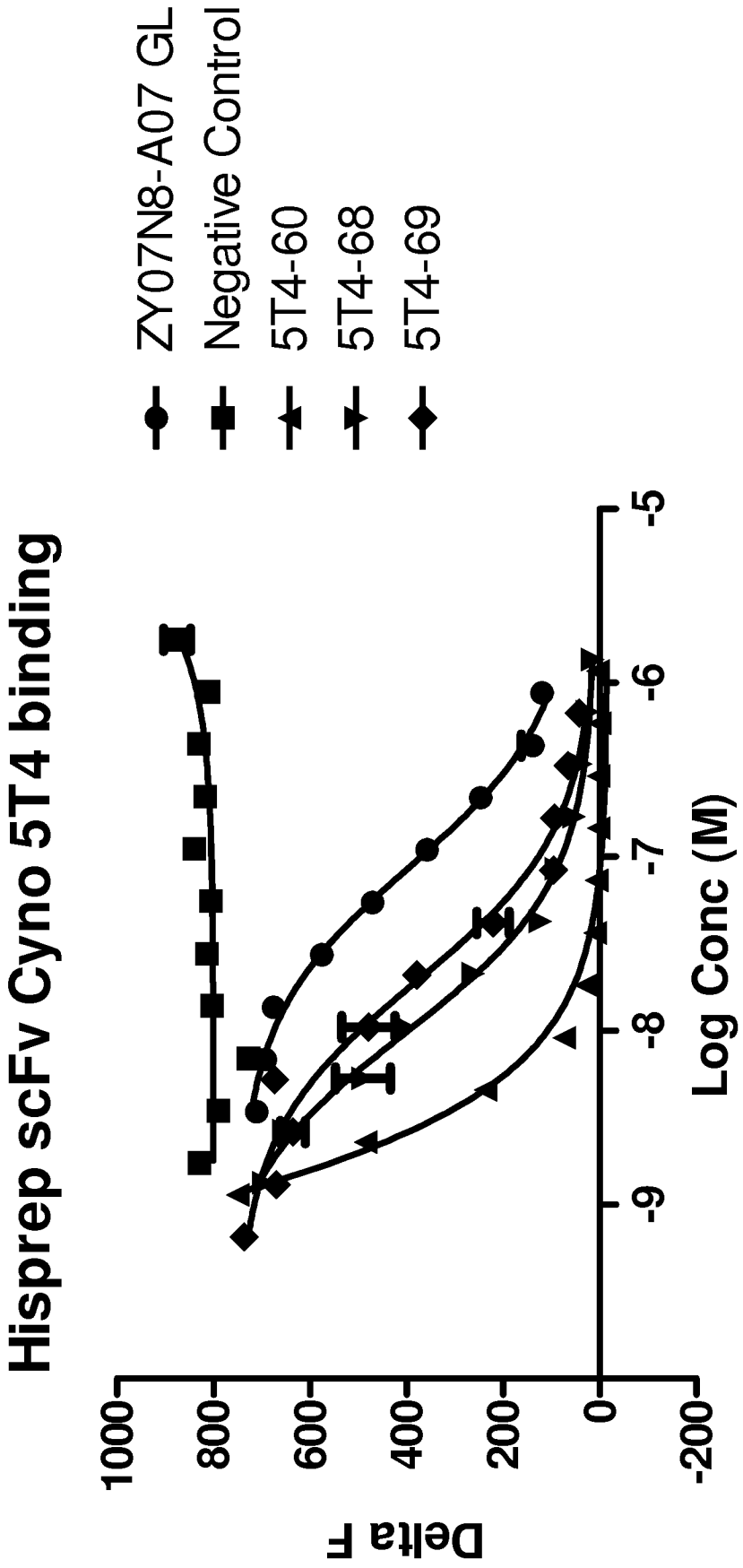

Hisprep scFv Cyno 5T4 binding
ZY07N8-A07 GL
Negative Control
5T4-60
5T4-68
5T4-69
Delta F
1000
800
600
400
200
0
-200
-5
-6
-7
-8
-9
Log Conc (M)

FIG. 9

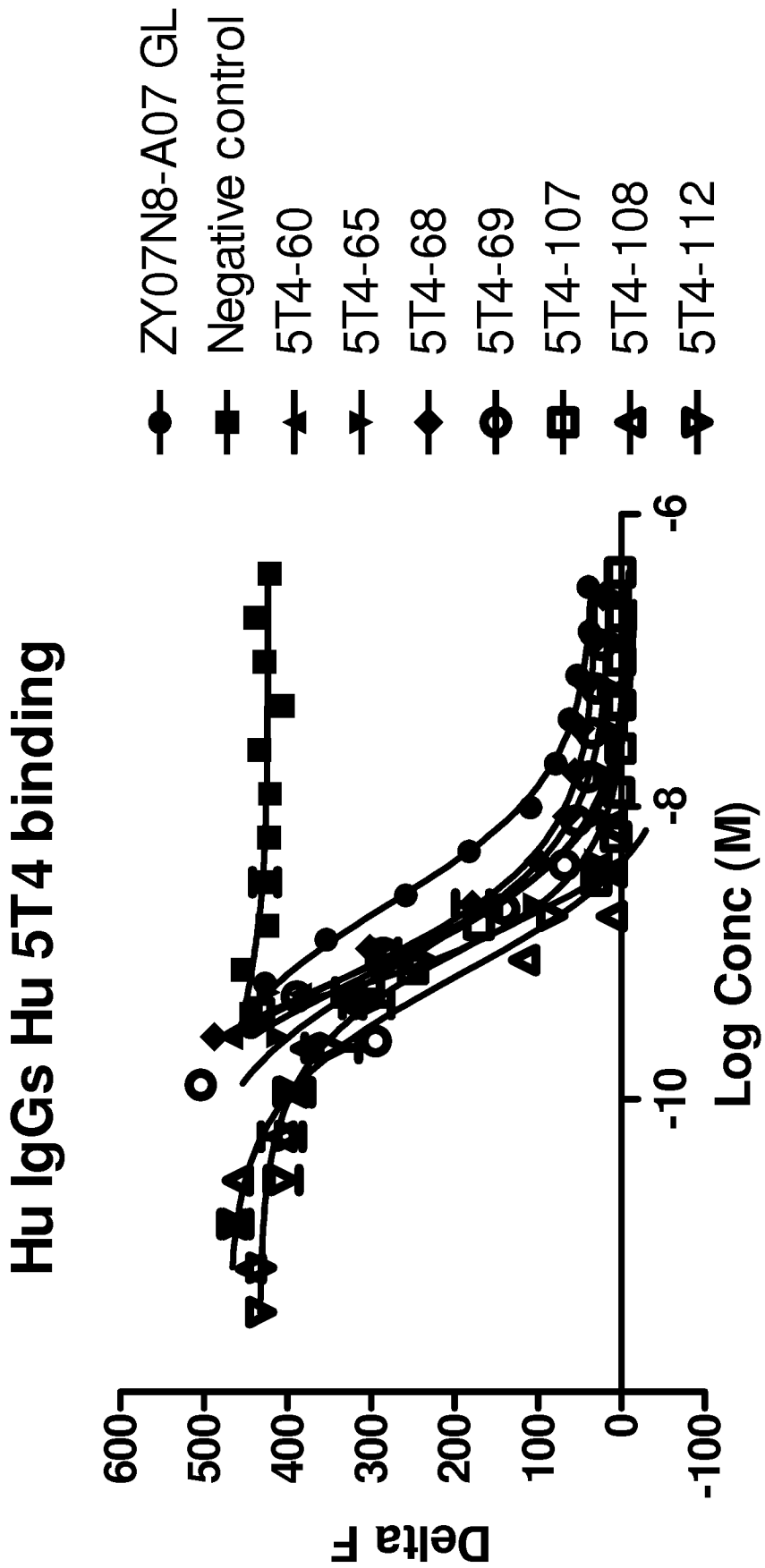

Hu IgGs Hu 5T4 binding
ZY07N8-A07 GL
Negative control
5T4-60
5T4-65
5T4-68
5T4-69
5T4-107
5T4-108
5T4-112
Delta F
600
500
400
300
200
100
0
-100
-10
-8
-6
Log Conc (M)

FIG. 10

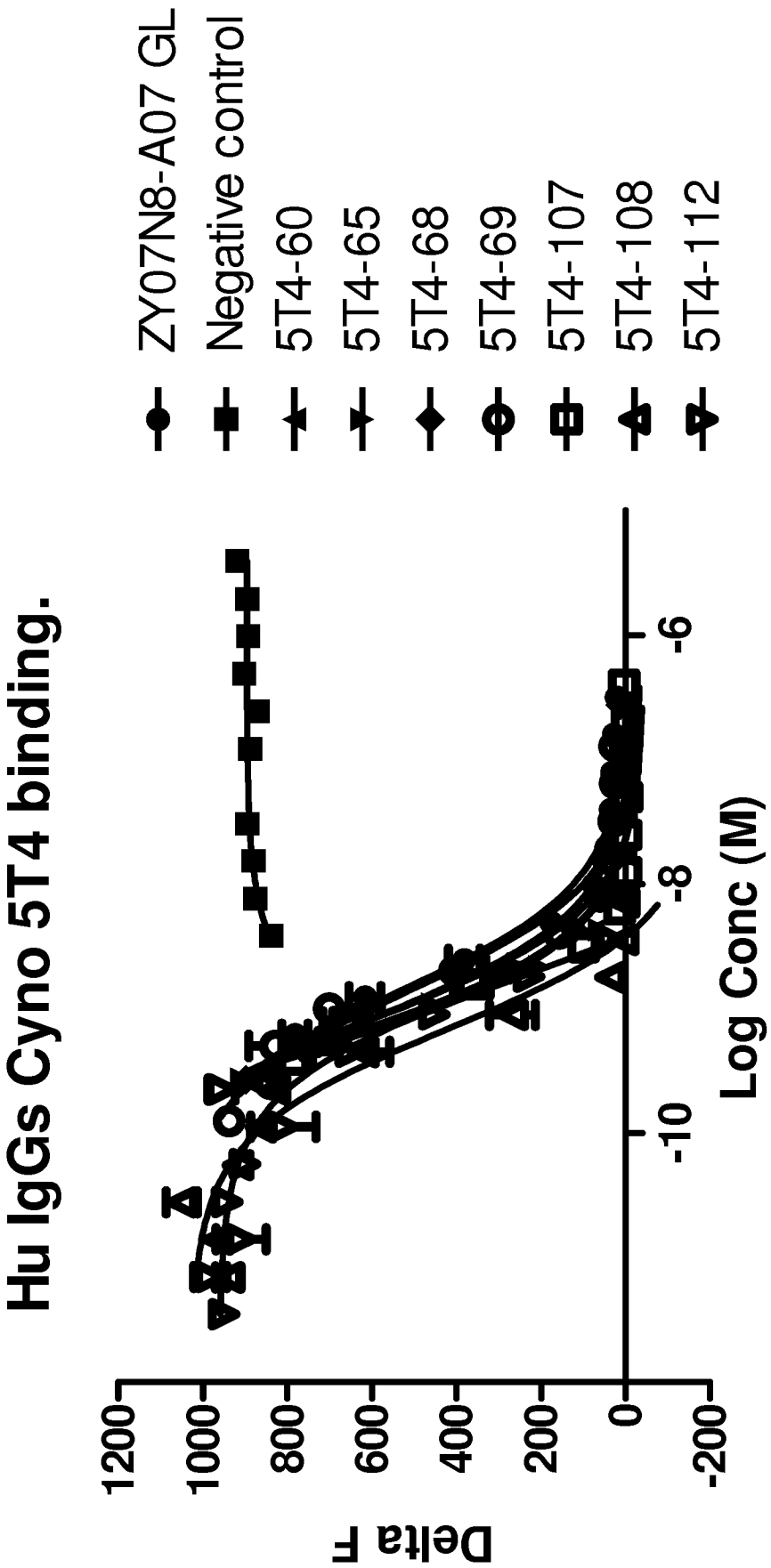
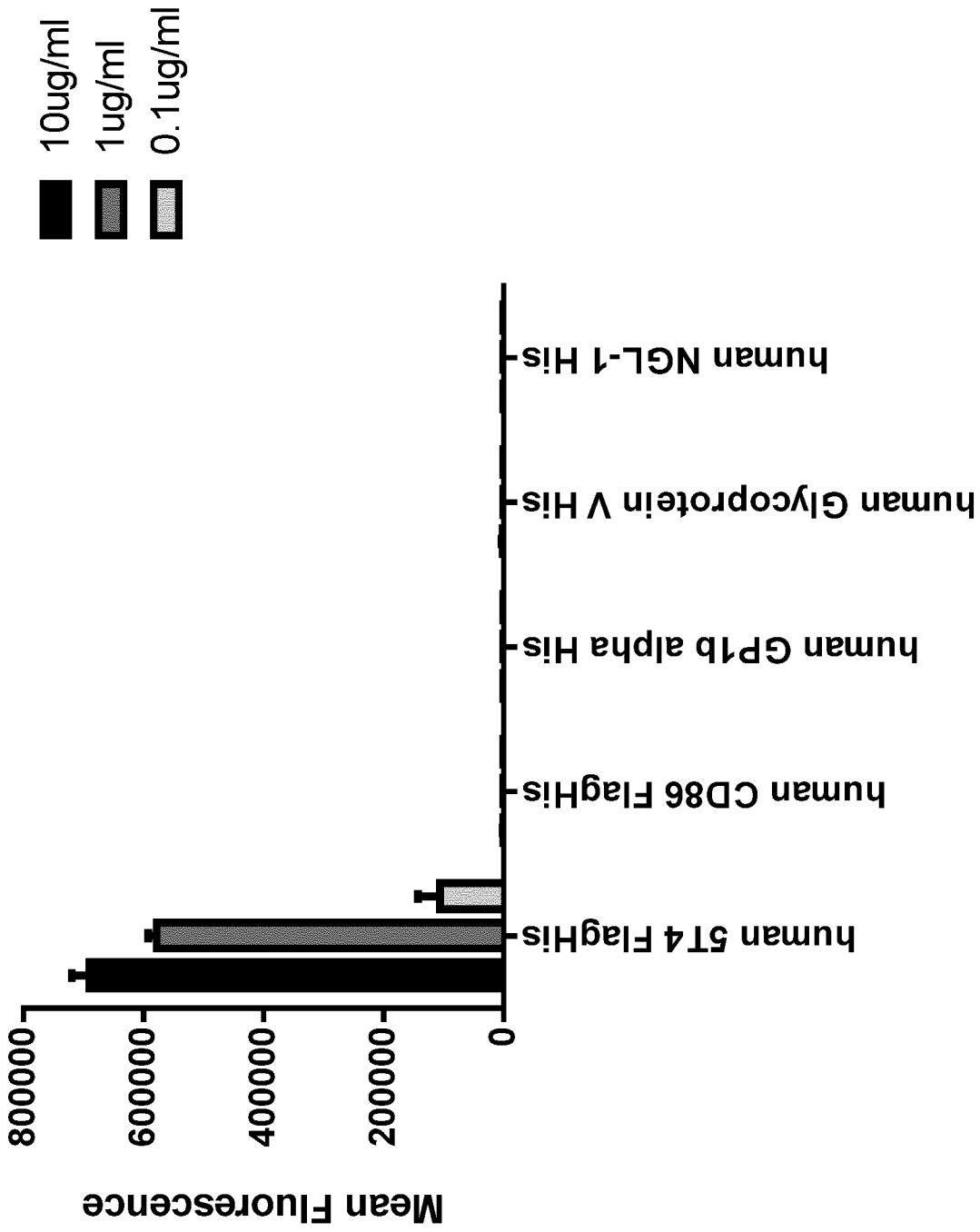
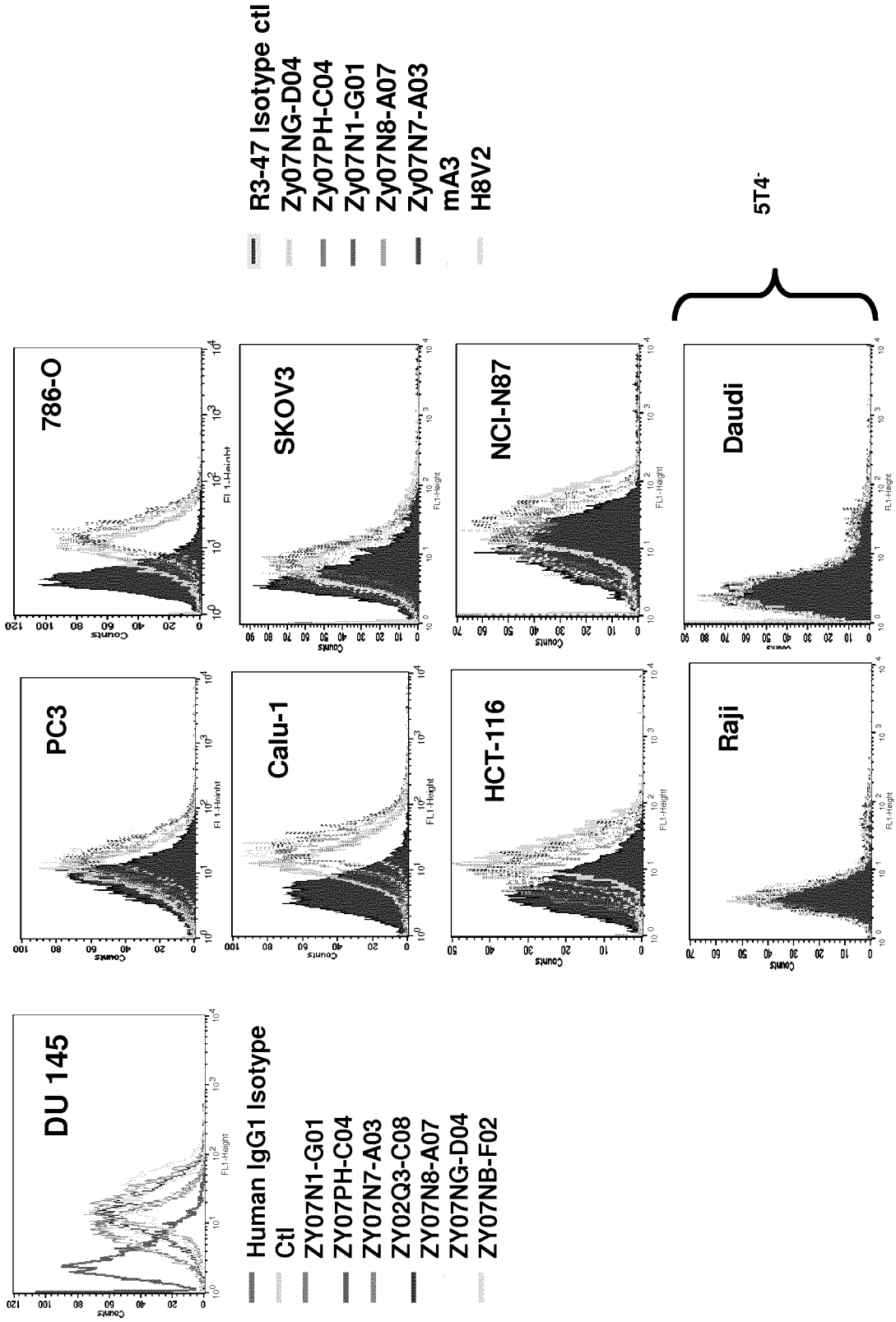

Hu IgGs Cyno 5T4 binding.
ZY07N8-A07 GL
Negative control
5T4-60
5T4-65
5T4-68
5T4-69
5T4-107
5T4-108
5T4-112
Delta F
1200
1000
800
600
400
200
0
-200
-10
-8
-6
Log Conc (M)

FIG. 11


10ug/ml
1ug/ml
0.1ug/ml
Mean Fluorescence
800000
600000
400000
200000
0
human 5T4 FlagHis
human CD86 FlagHis
human GP1b alpha His
human Glycoprotein V His
human NGL-1 His

FIGS. 12A-I


R3-47 Isotype ctl
Zy07NG-D04
Zy07PH-C04
Zy07N1-G01
Zy07N8-A07
Zy07N7-A03
mA3
H8V2
5T4-
786-O
SKOV3
NCI-N87
Daudi
PC3
Calu-1
HCT-116
Raji
DU 145
Human IgG1 Isotype
Ctl
ZY07N1-G01
ZY07PH-C04
ZY07N7-A03
ZY02Q3-C08
ZY07N8-A07
ZY07NG-D04
ZY07NB-F02
Counts
FL1-Height

FIGS. 13A-J

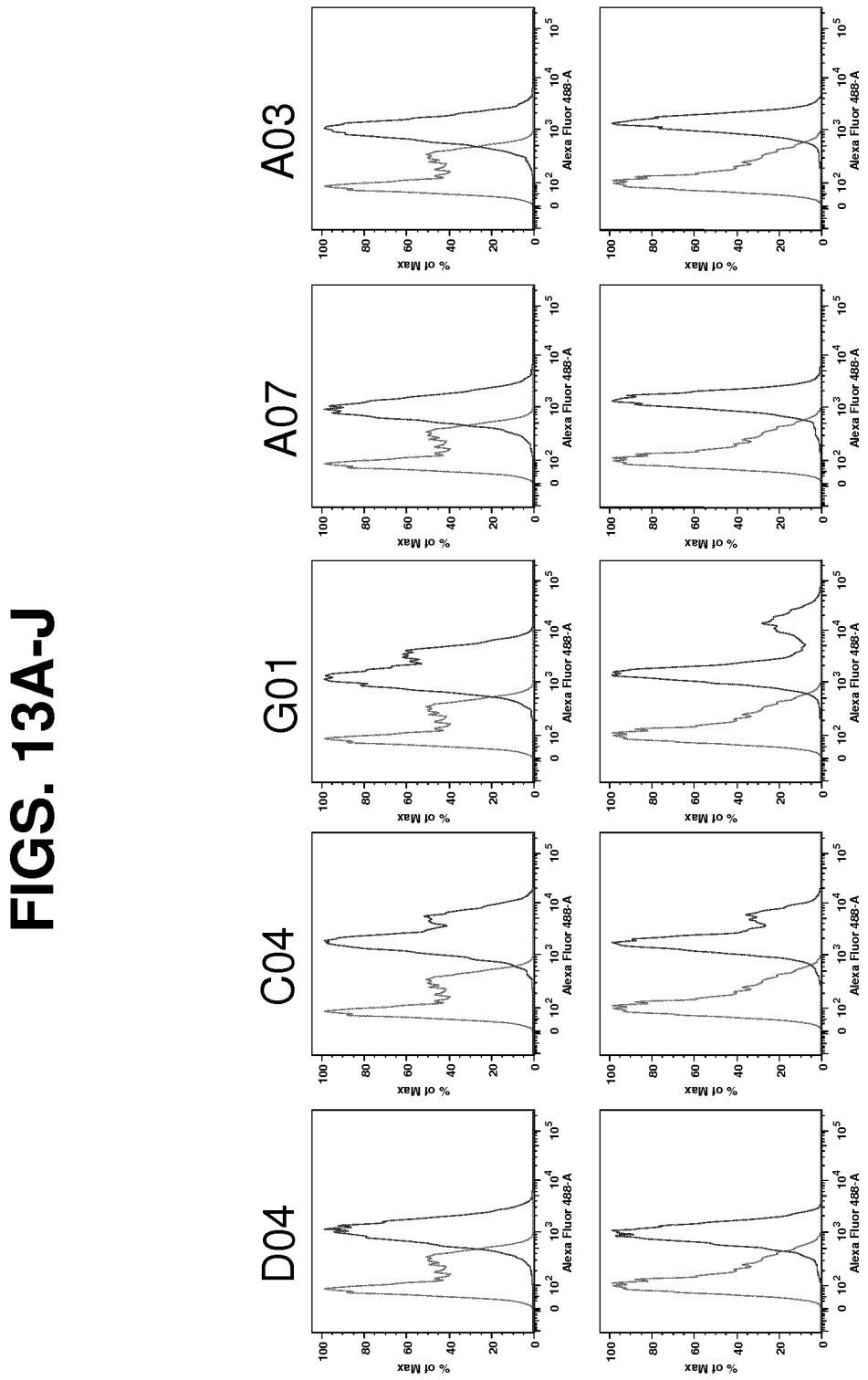

D04
C04
G01
A07
A03
DU 145 (Human)
MK-1 (Cyno)
% of Max
Alexa Fluor 488-A
Red curve: isotype control
Blue curve: 5T4 Ab's

FIG. 14A

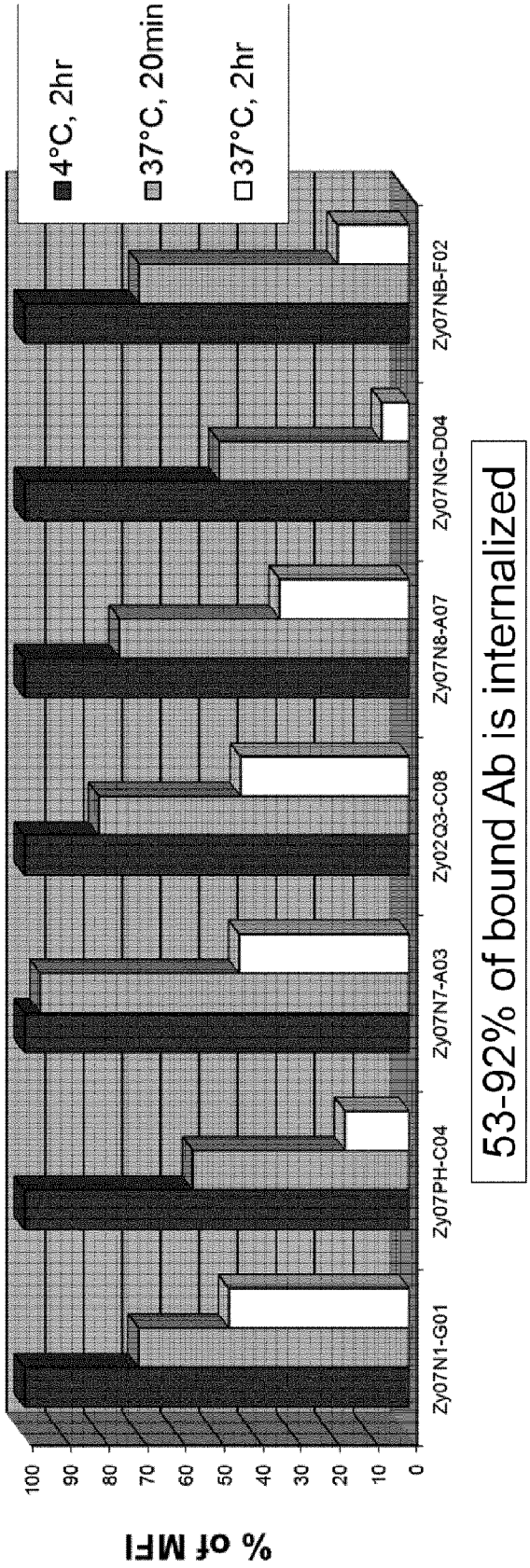

4°C, 2hr
37°C, 20min
37°C, 2hr
% of MFI
100
90
80
70
60
50
40
30
20
10
0
Zy07N1-G01
Zy07PH-C04
Zy07N7-A03
Zy02Q3-C08
Zy07N8-A07
Zy07NG-D04
Zy07NB-F02
53-92% of bound Ab is internalized

FIG. 14B

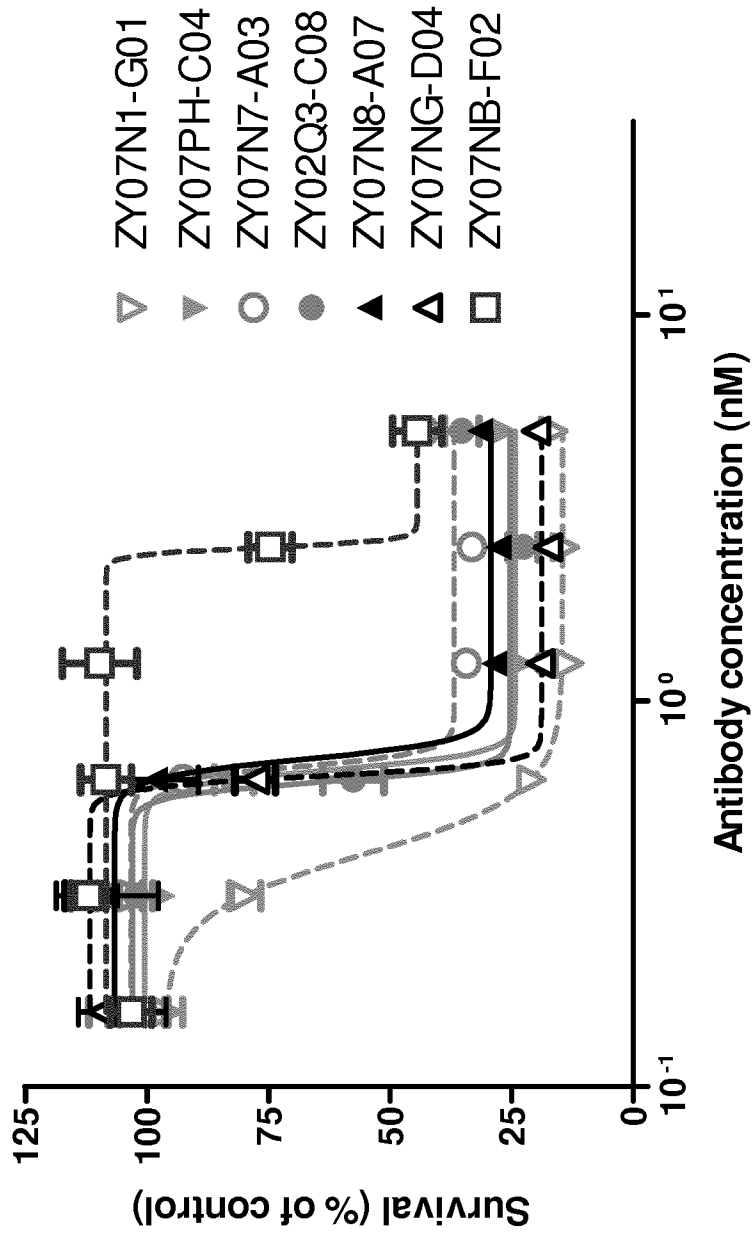

ZY07N1-G01
ZY07PH-C04
ZY07N7-A03
ZY02Q3-C08
ZY07N8-A07
ZY07NG-D04
ZY07NB-F02
Survival (% of control)
125
100
75
50
25
0
10^{-1}
10^{0}
10^{1}
Antibody concentration (nM)

FIG. 15

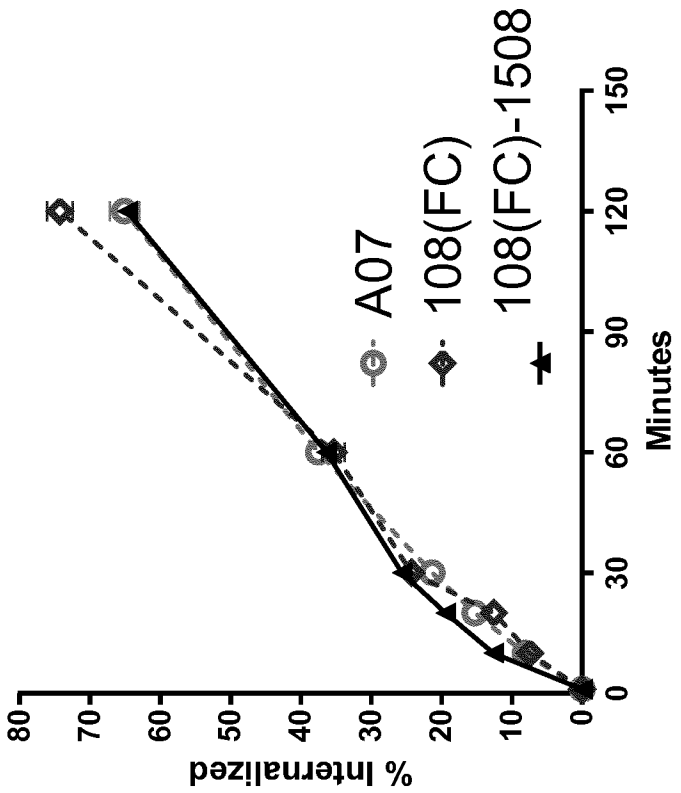

% Internalized
0
10
20
30
40
50
60
70
80
0
30
60
90
120
150
Minutes
A07
108(FC)
108(FC)-1508

FIG. 16

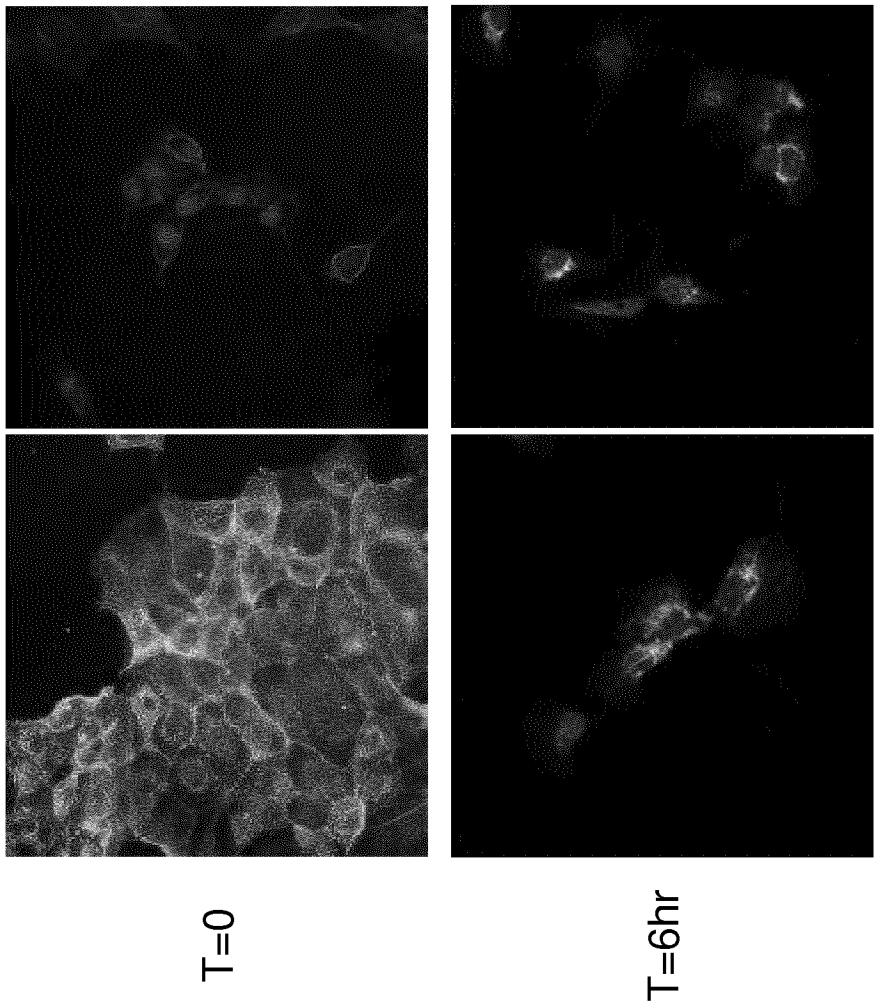


FIG. 17A

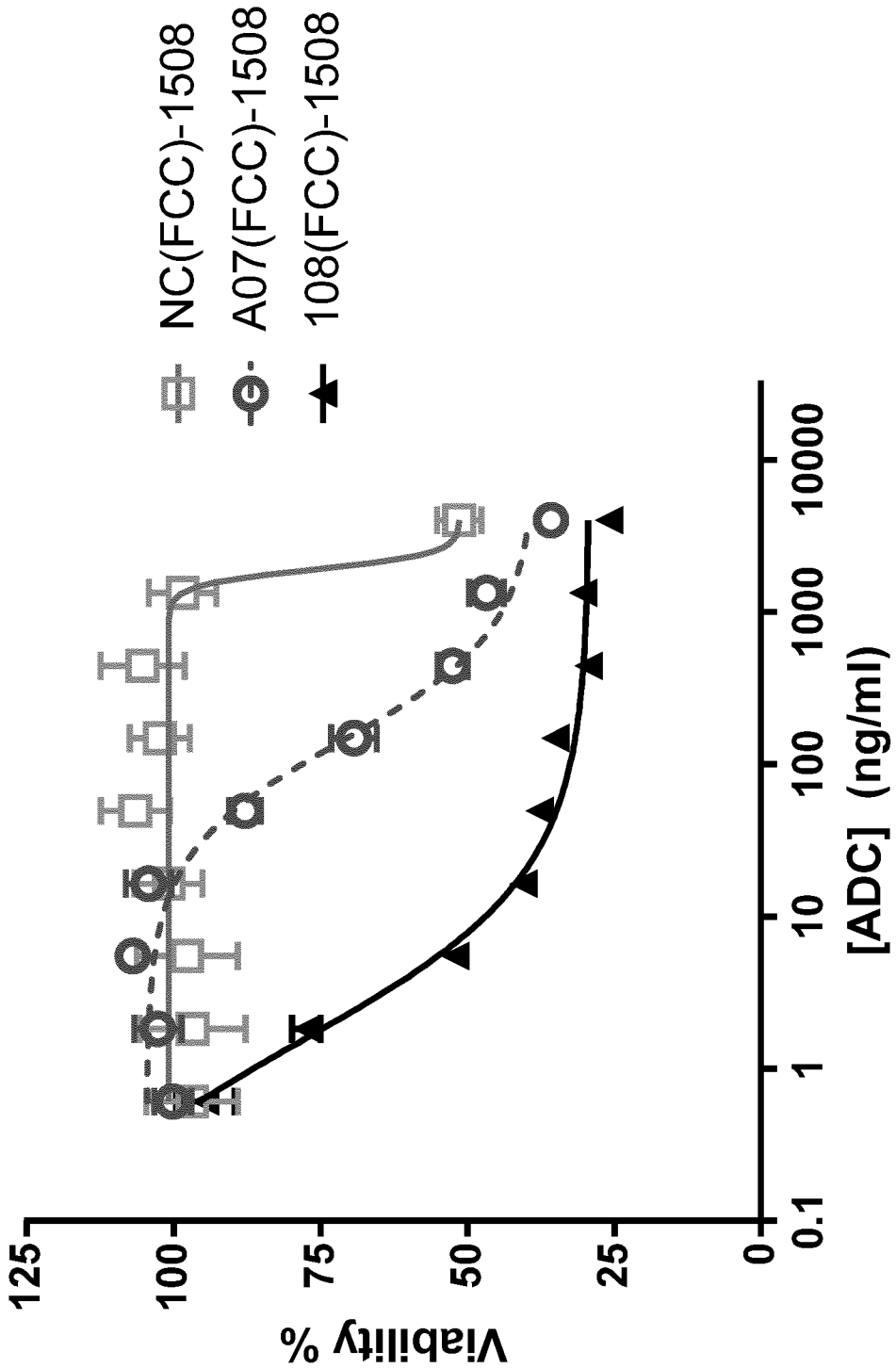
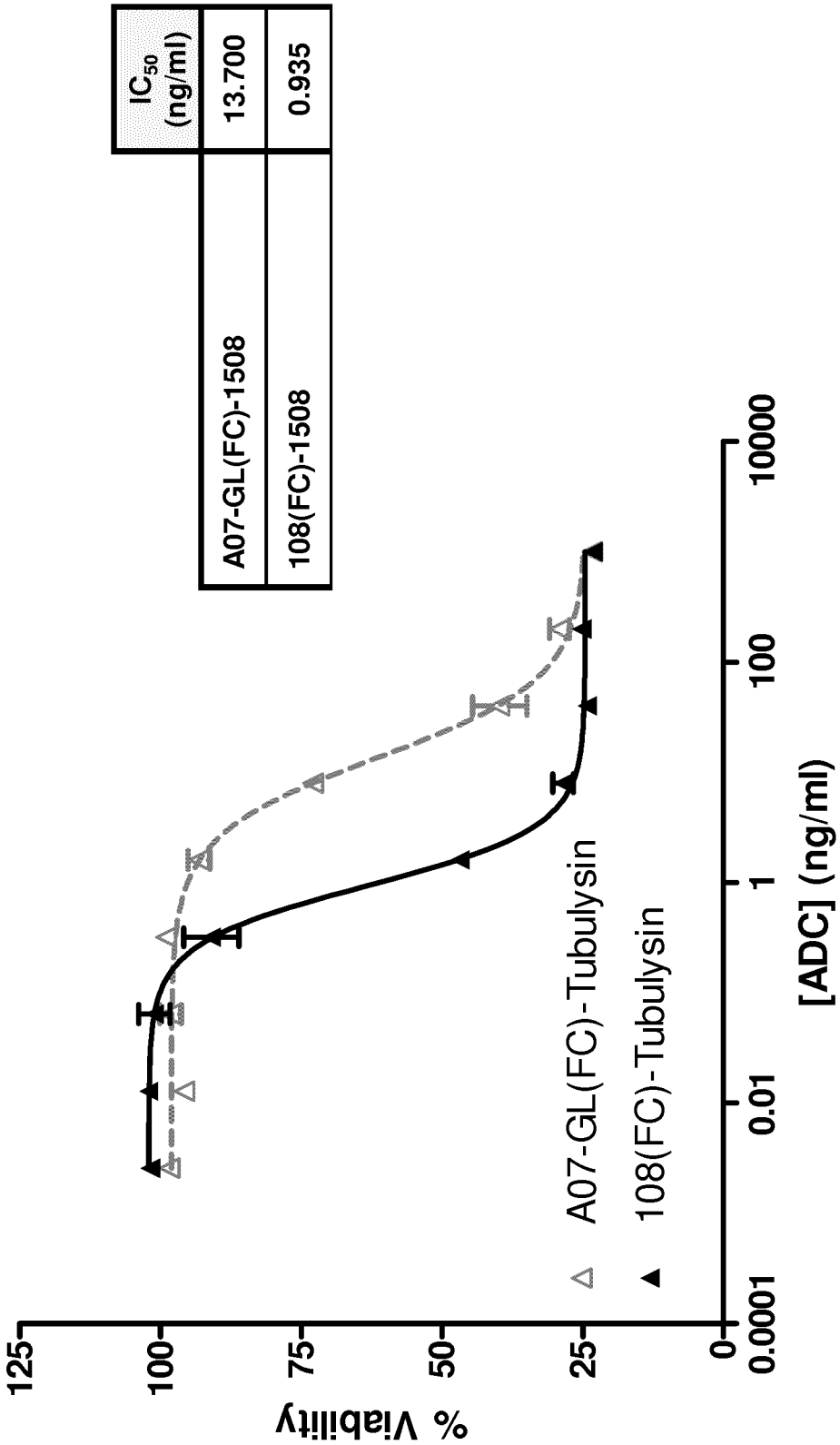

NC(FCC)-1508
A07(FCC)-1508
108(FCC)-1508
Viability %
125
100
75
50
25
0
0.1
1
10
100
1000
10000
[ADC] (ng/ml)

FIG. 17B


IC50 (ng/ml)
A07-GL(FC)-1508 13.700
108(FC)-1508 0.935
% Viability
125
100
75
50
25
0
0.0001
0.01
1
100
10000
[ADC] (ng/ml)
A07-GL(FC)-Tubulysin
108(FC)-Tubulysin

FIGS. 18A-B

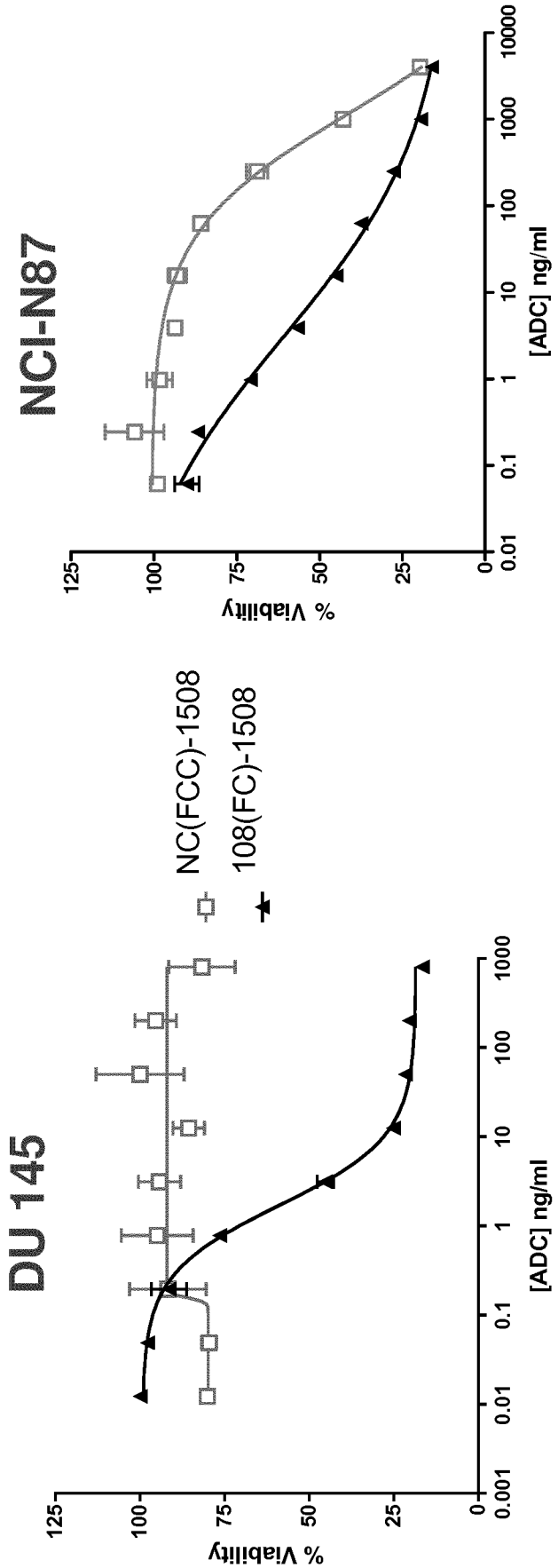
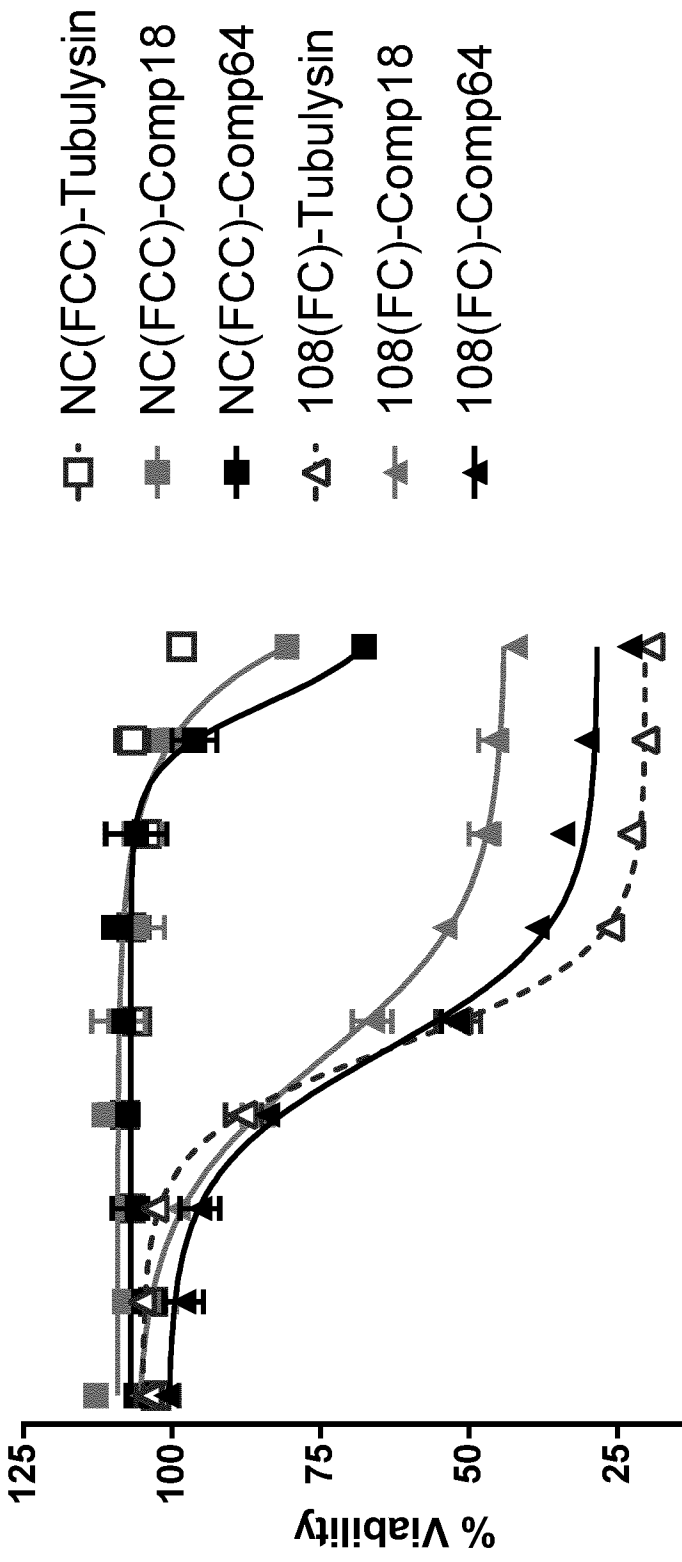

NCI-N87
% Viability
[ADC] ng/ml
0
25
50
75
100
125
0.01
0.1
1
10
100
1000
10000
DU 145
NC(FCC)-1508
108(FC)-1508
% Viability
[ADC] ng/ml
0
25
50
75
100
125
0.001
0.01
0.1
1
10
100
1000

FIG. 19A



	IC_{50} (ng/ml)	Max Kill (%)
108(FC)-Tubulysin	2.669	80.5
108(FC)-Comp18	2.192	57.7
108(FC)-Comp64	2.471	77.0

FIG. 19B

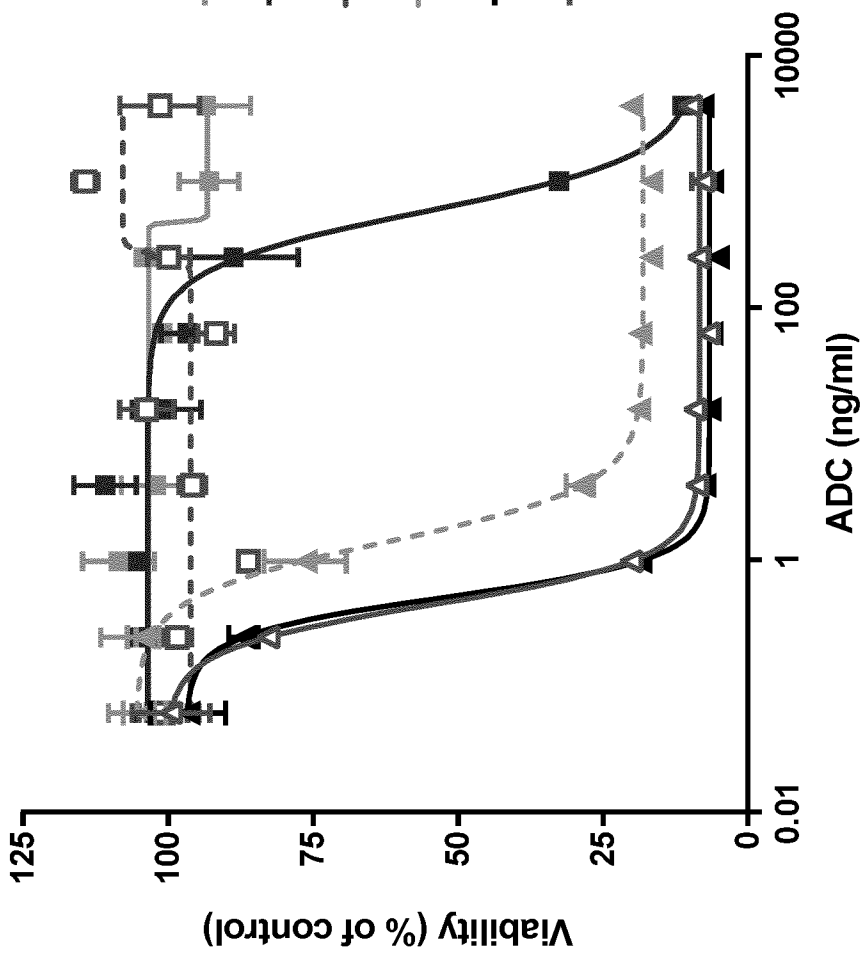

NC(FC)-1508
NC(FC)-Comp64
NC(FC)-Comp96
108(FC)-1508 (IC50=1.41ng/ml)
108(FC)-Comp64 (IC50=0.51ng/ml)
108(FC)-Comp96 (IC50=0.44ng/ml)
Viability (% of control)
125
100
75
50
25
0
0.01
1
100
10000
ADC (ng/ml)

FIG. 20

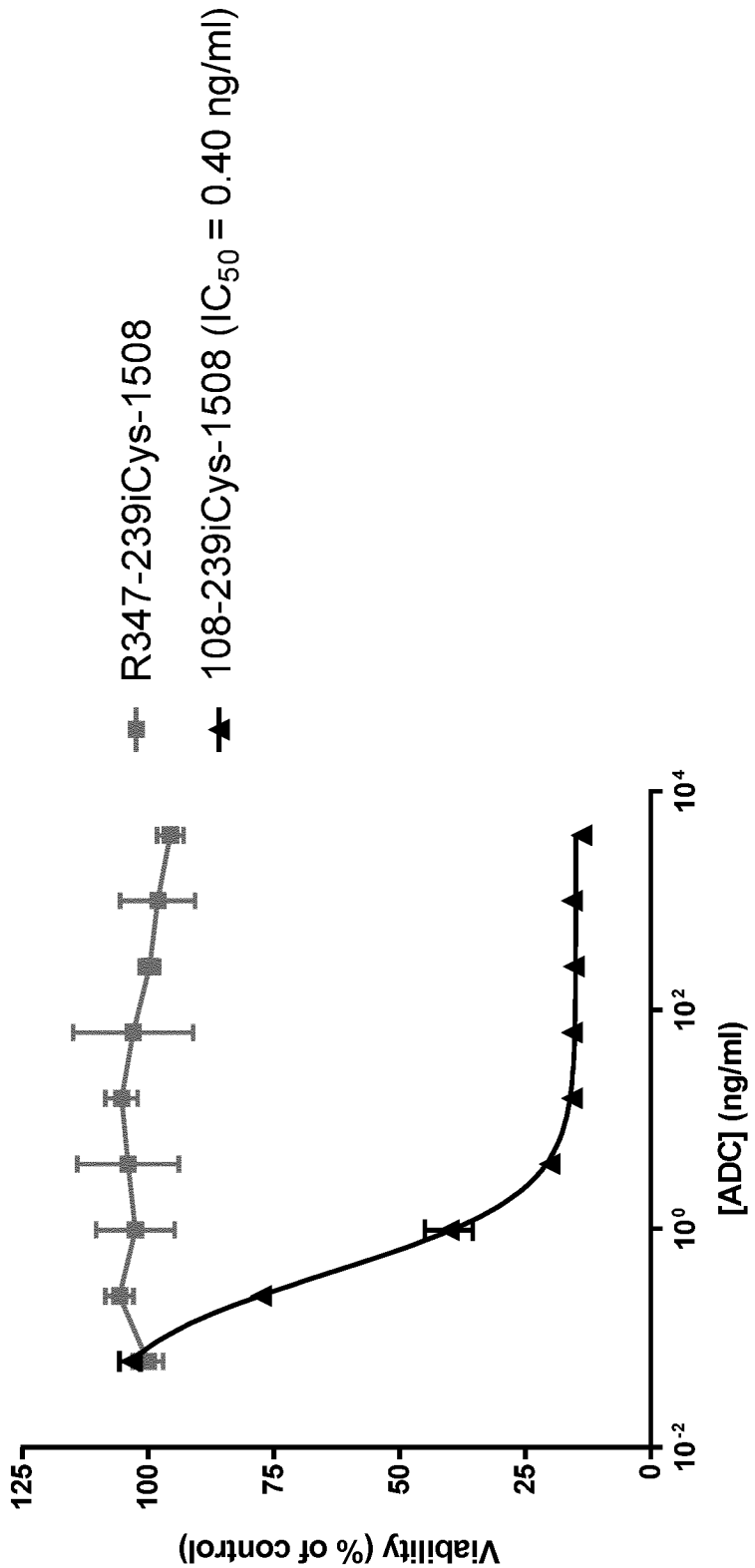

R347-239iCys-1508
108-239iCys-1508 (IC50 = 0.40 ng/ml)
Viability (% of control)
125
100
75
50
25
0
10-2
100
102
104
[ADC] (ng/ml)

FIG. 21

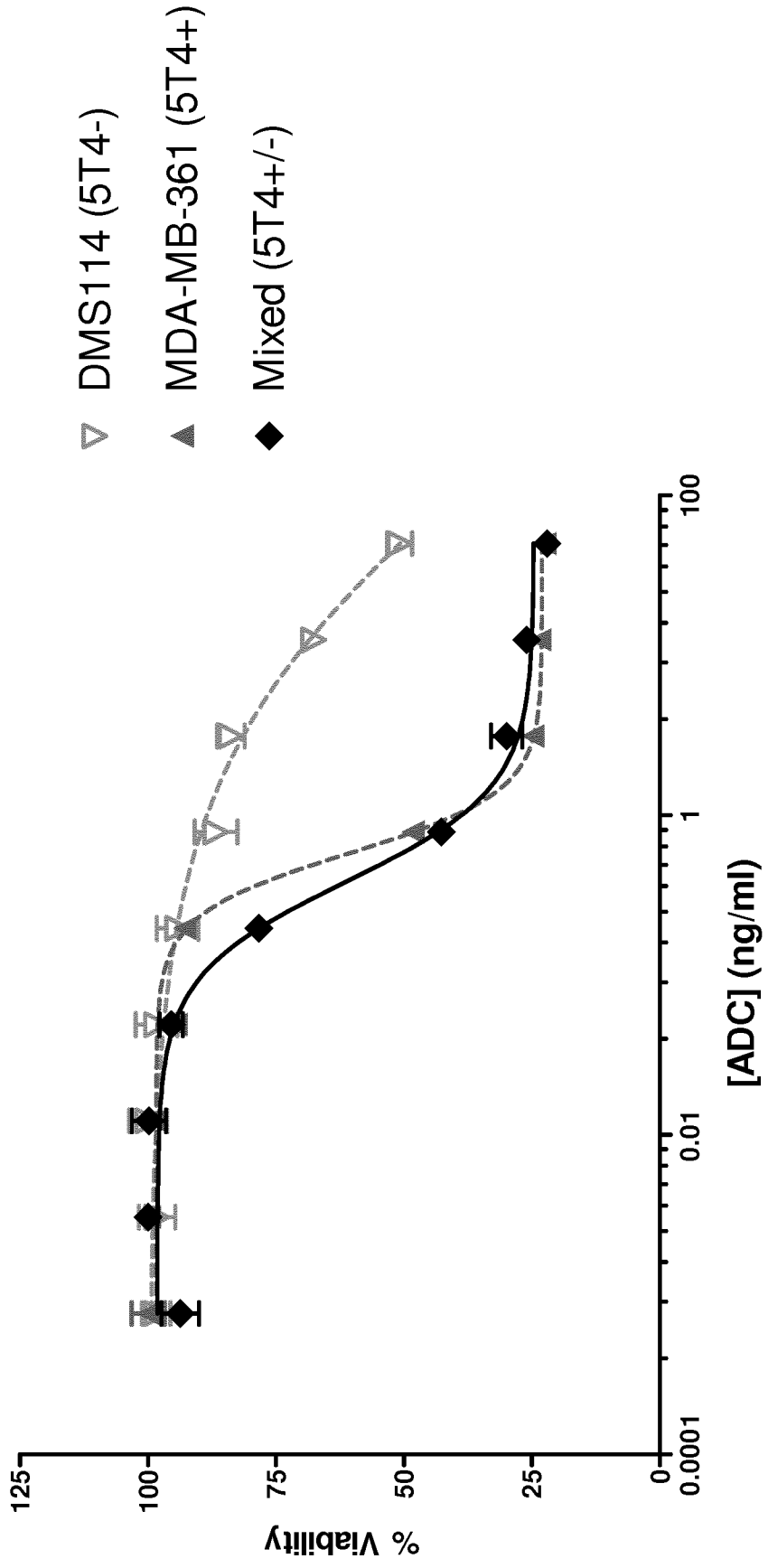

DMS114 (5T4-)
MDA-MB-361 (5T4+)
Mixed (5T4+/-)
% Viability
125
100
75
50
25
0
0.0001
0.01
1
100
[ADC] (ng/ml)

FIG. 22

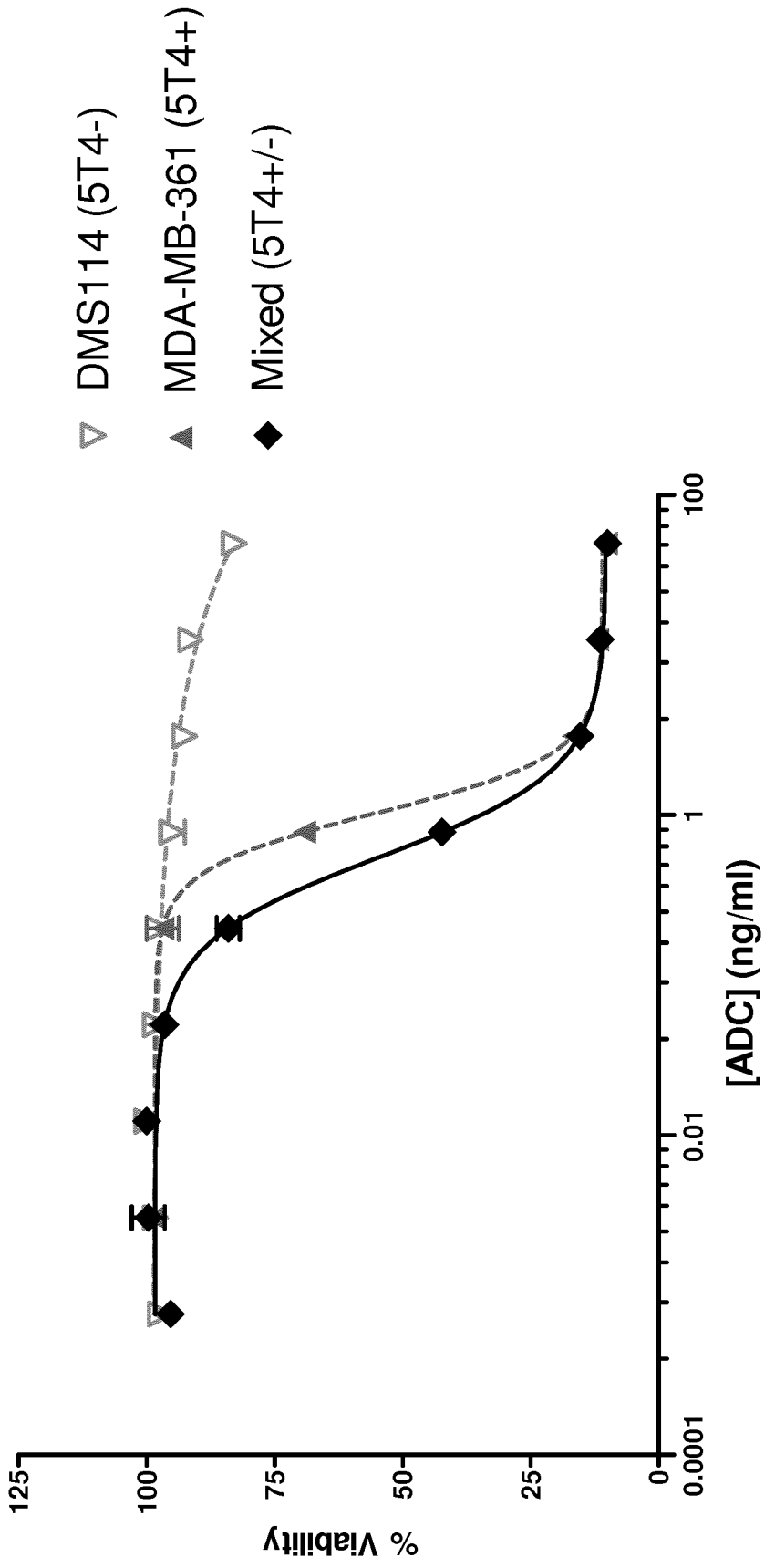

DMS114 (5T4-)
MDA-MB-361 (5T4+)
Mixed (5T4+/-)
% Viability
125
100
75
50
25
0
0.0001
0.01
1
100
[ADC] (ng/ml)

FIG. 23

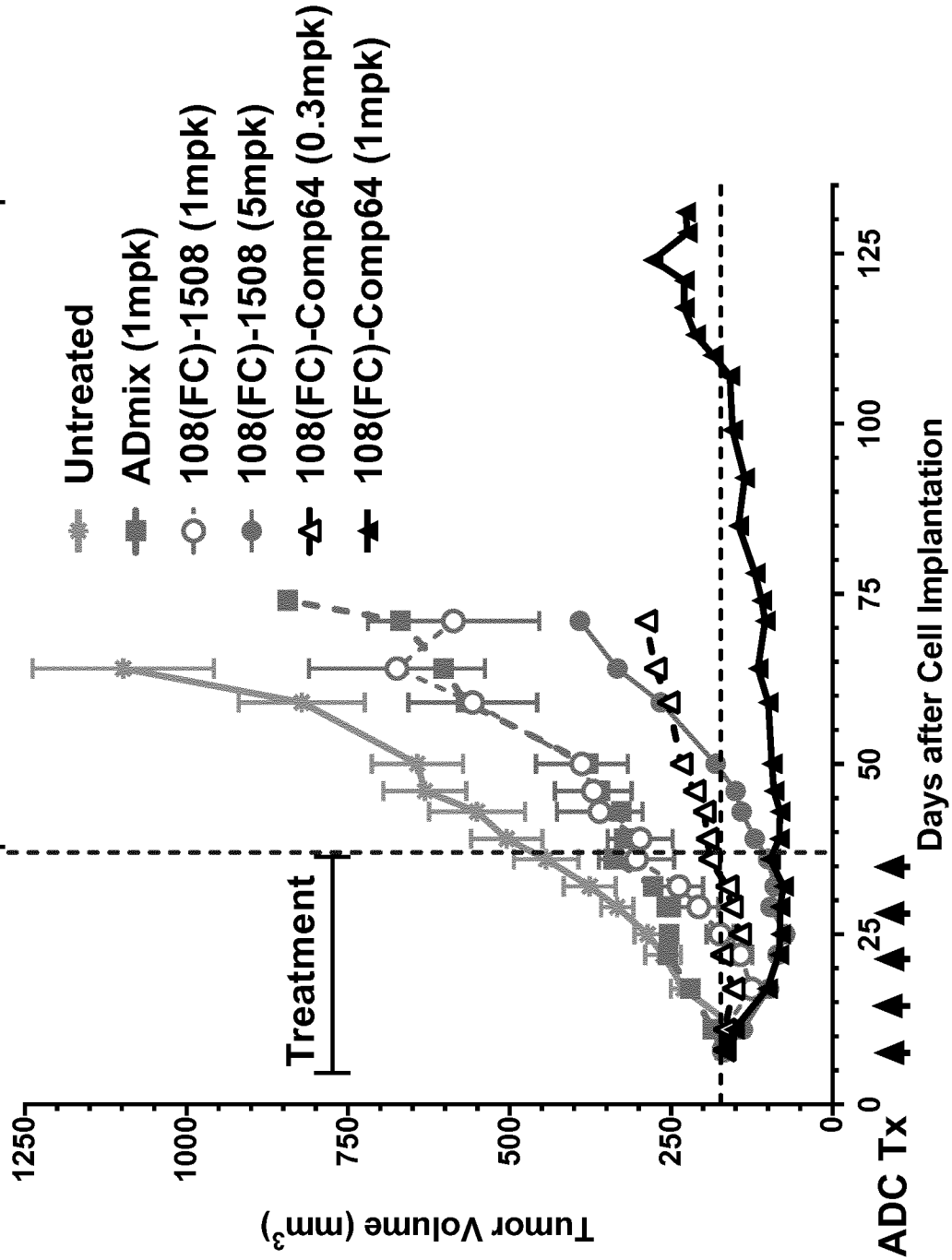

Regrowth
Treatment
Untreated
ADmix (1mpk)
108(FC)-1508 (1mpk)
108(FC)-1508 (5mpk)
108(FC)-Comp64 (0.3mpk)
108(FC)-Comp64 (1mpk)
Tumor Volume (mm3)
1250
1000
750
500
250
0
ADC Tx
0
25
50
75
100
125
Days after Cell Implantation

FIG. 24

	ΔTGI
NC(FC)-Comp18(1mpk)	52%
108(FC)-Comp18(0.3mpk)	92.7%
108(FC)-Comp64(0.3mpk)	71.6%
108(FC)-Comp18(1mpk)	190.4%
108(FC)-Comp64 (1mpk)	154.4%

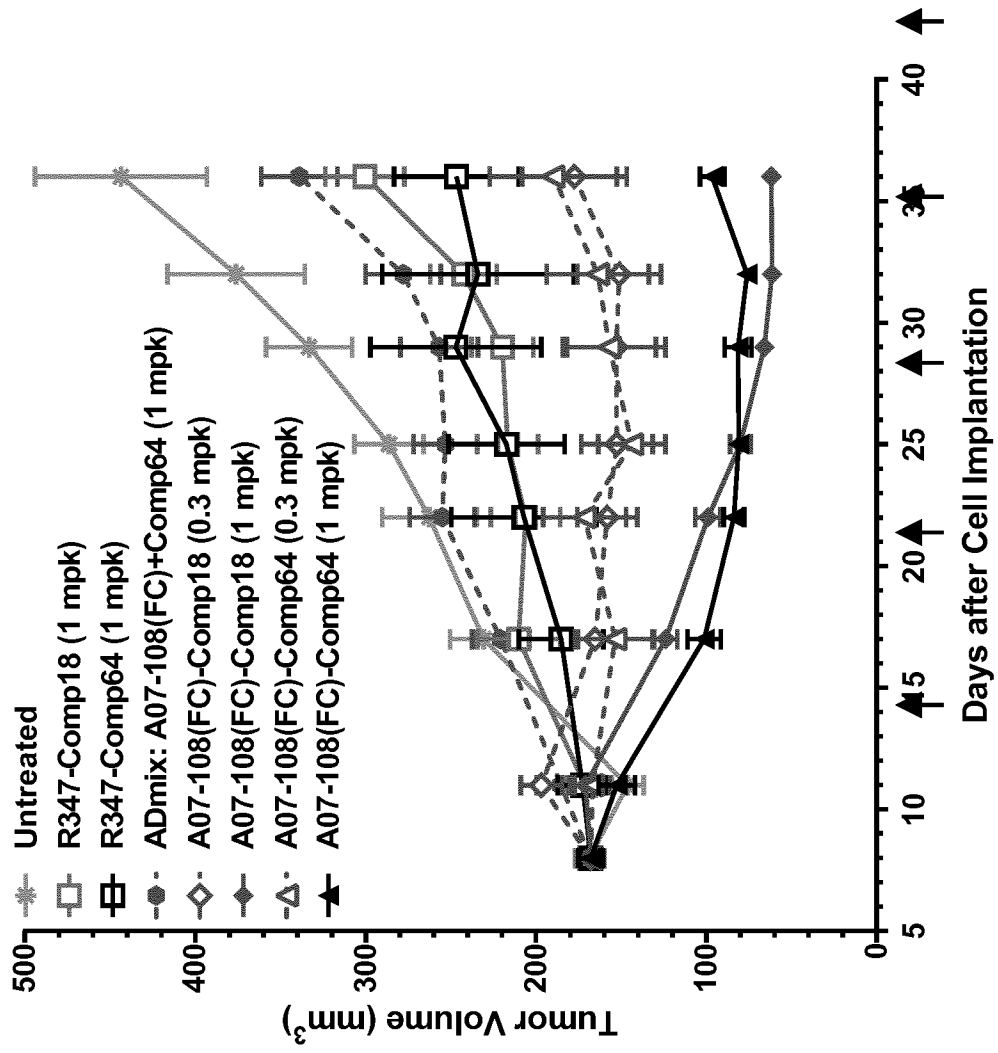


FIG. 25A

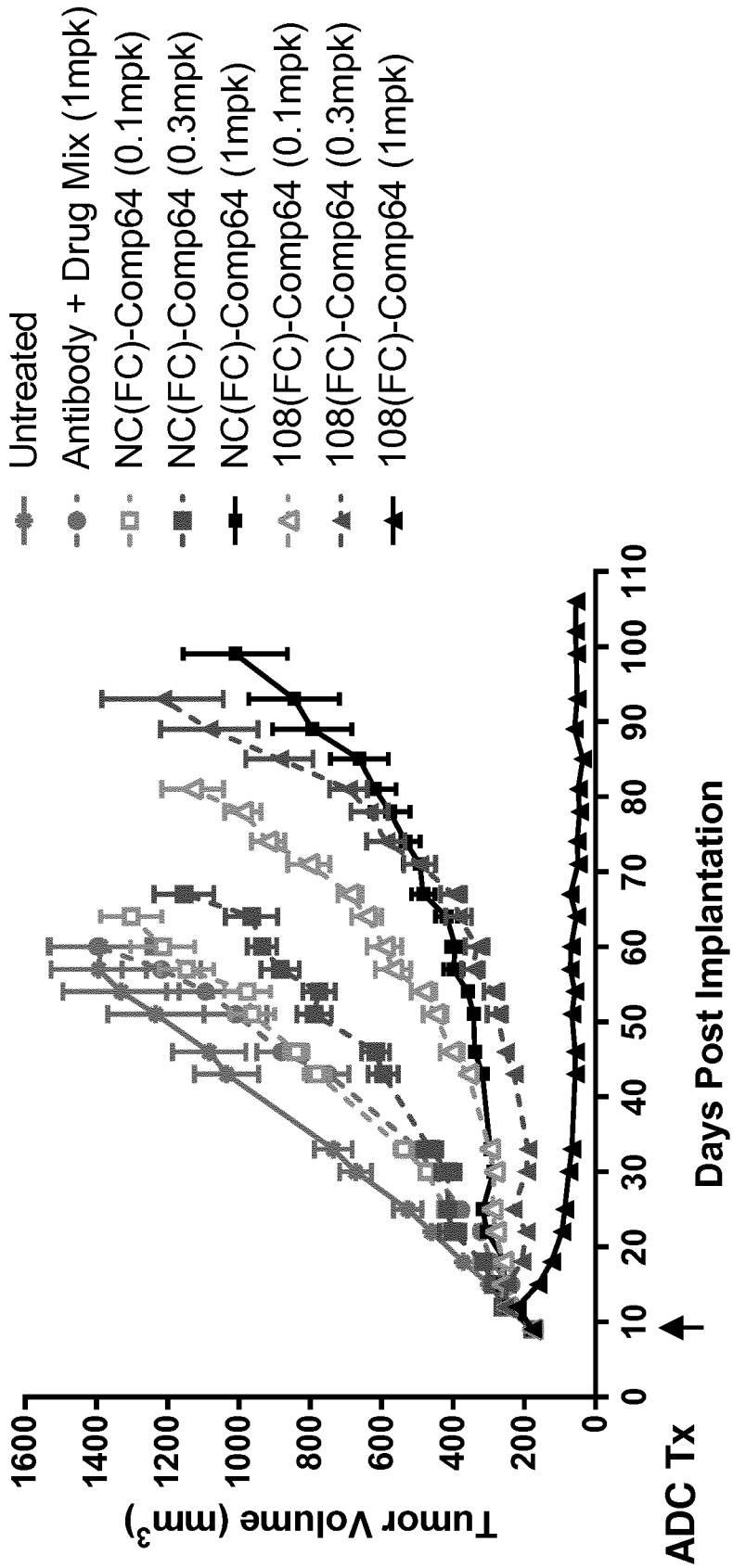

Untreated
Antibody + Drug Mix (1mpk)
NC(FC)-Comp64 (0.1mpk)
NC(FC)-Comp64 (0.3mpk)
NC(FC)-Comp64 (1mpk)
108(FC)-Comp64 (0.1mpk)
108(FC)-Comp64 (0.3mpk)
108(FC)-Comp64 (1mpk)
Tumor Volume (mm3)
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
80
90
100
110
ADC Tx
Days Post Implantation

FIG. 25B

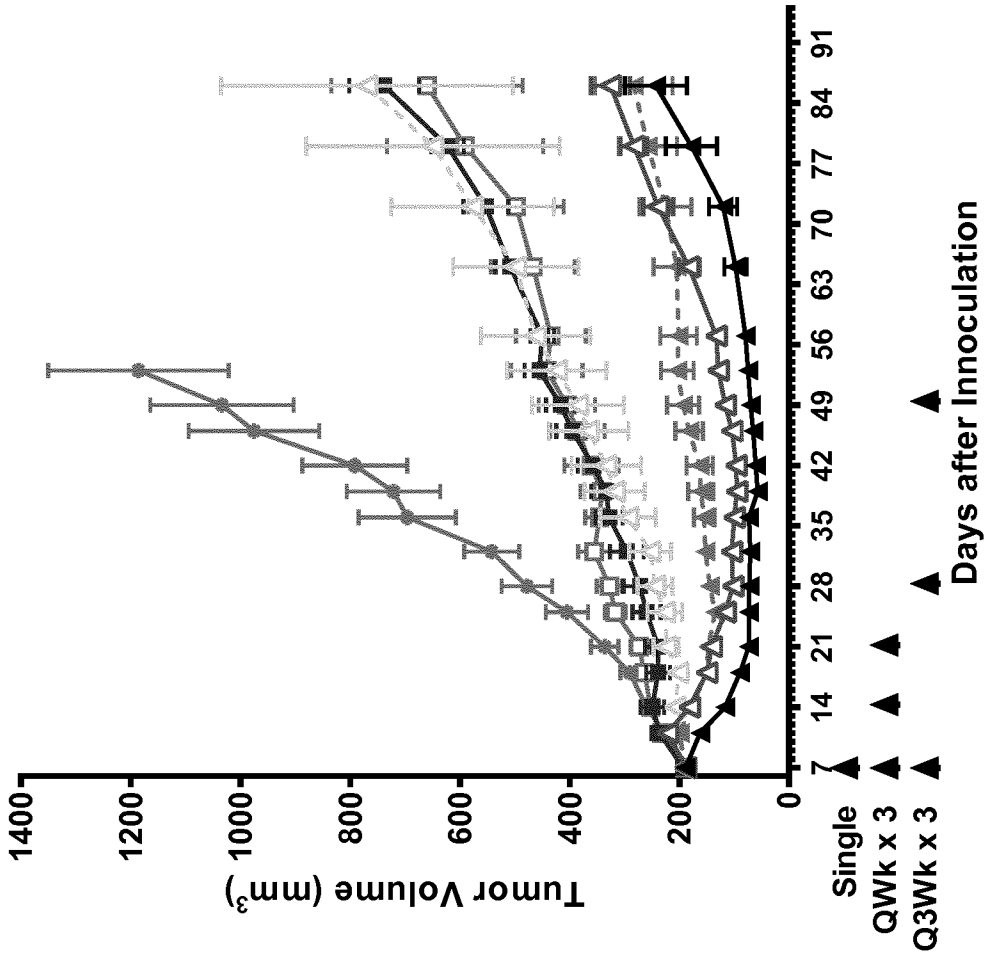

Untreated
NC(FC)-Comp64: 1mpk (Single)
NC(FC)-Comp64: 0.33mpk (QWk x 3)
108(FC)-Comp64: 0.1mpk (Q3Wk x 3)
108(FC)-Comp64: 0.33mpk (Q3Wk x 3)
108(FC)-Comp64: 0.33mpk (QWk x 3)
108(FC)-Comp64: 1mpk (Single)
Tumor Volume (mm3)
1400
1200
1000
800
600
400
200
0
7 14 21 28 35 42 49 56 63 70 77 84 91
Days after Innoculation
Single
QWk x 3
Q3Wk x 3

FIG. 25C

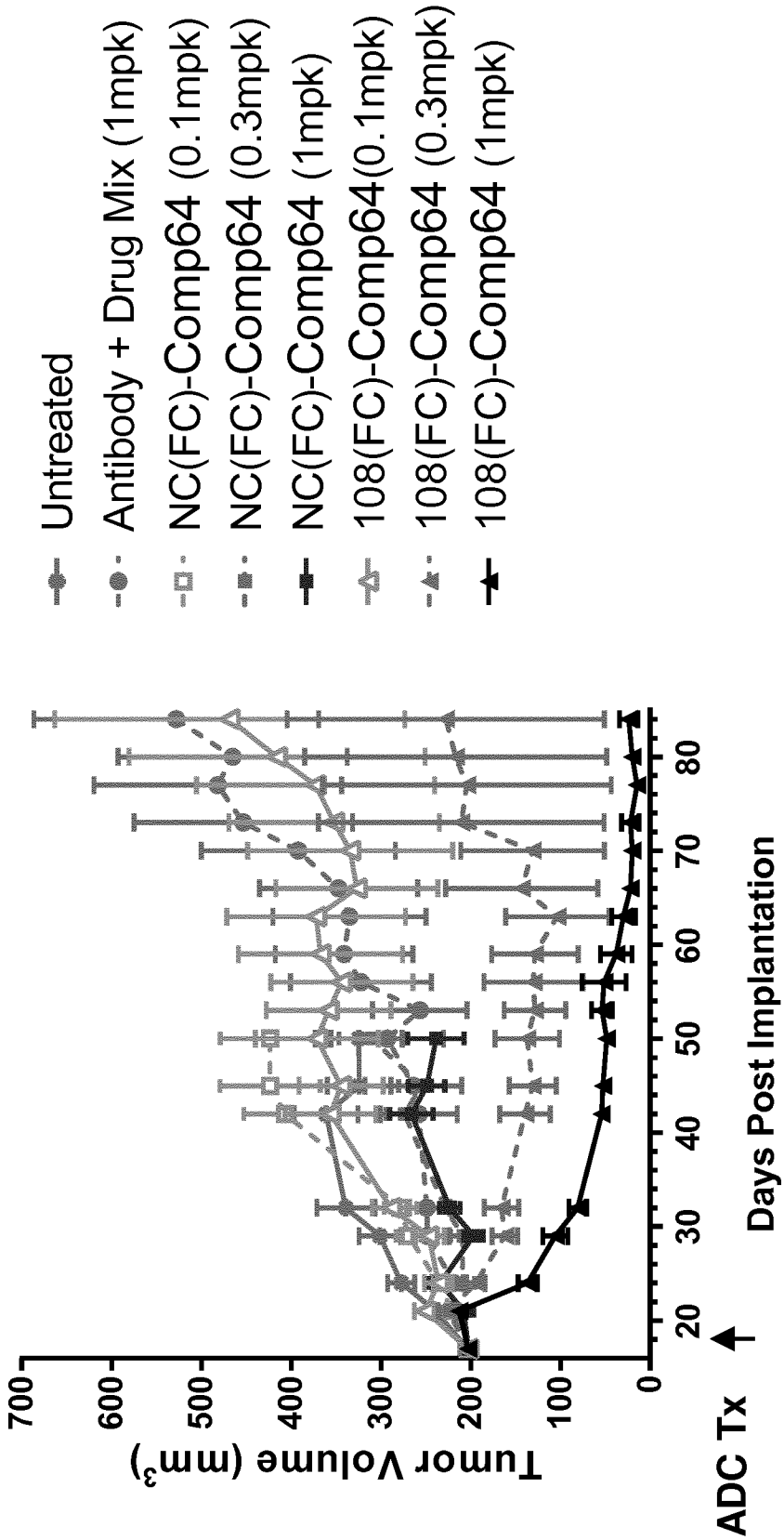

Untreated
Antibody + Drug Mix (1mpk)
NC(FC)-Comp64 (0.1mpk)
NC(FC)-Comp64 (0.3mpk)
NC(FC)-Comp64 (1mpk)
108(FC)-Comp64(0.1mpk)
108(FC)-Comp64 (0.3mpk)
108(FC)-Comp64 (1mpk)
Tumor Volume (mm3)
0
100
200
300
400
500
600
700
ADC Tx
20
30
40
50
60
70
80
Days Post Implantation

FIG. 25D

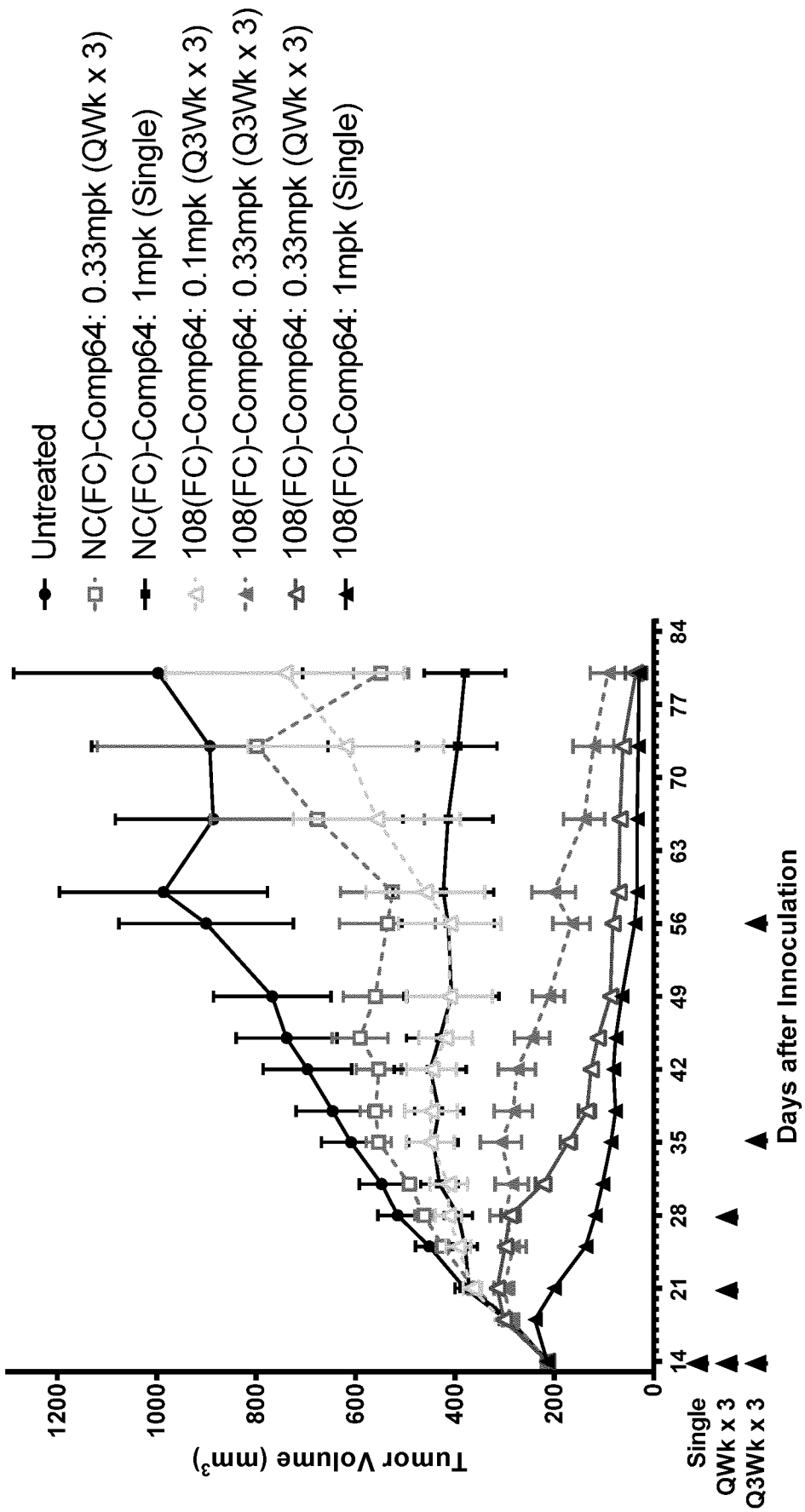

Untreated
NC(FC)-Comp64: 0.33mpk (QWk x 3)
NC(FC)-Comp64: 1mpk (Single)
108(FC)-Comp64: 0.1mpk (Q3Wk x 3)
108(FC)-Comp64: 0.33mpk (Q3Wk x 3)
108(FC)-Comp64: 0.33mpk (QWk x 3)
108(FC)-Comp64: 1mpk (Single)
Tumor Volume (mm3)
1200
1000
800
600
400
200
0
14
21
28
35
42
49
56
63
70
77
84
Days after Innoculation
Single
QWk x 3
Q3Wk x 3

FIG. 26A

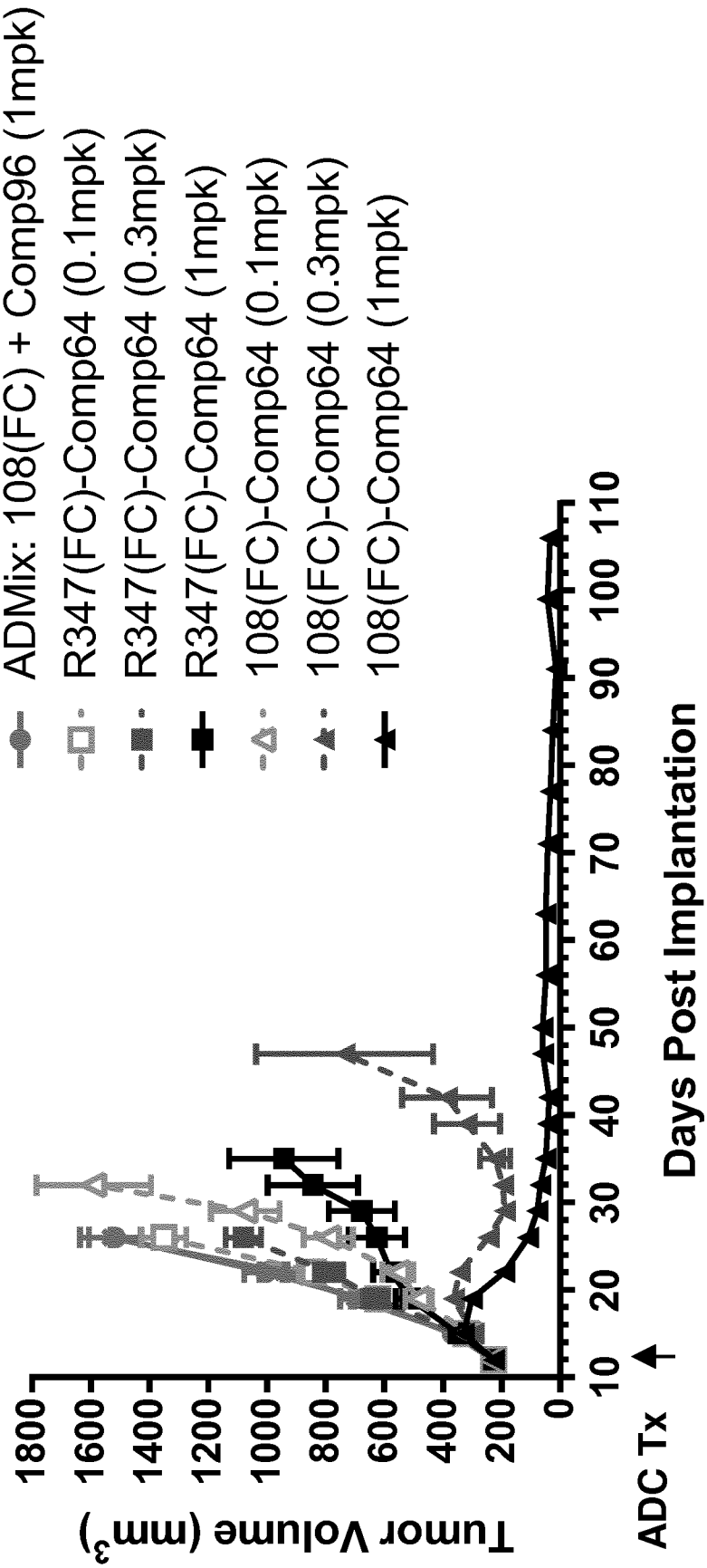

Vehicle
ADMix: 108(FC) + Comp96 (1mpk)
R347(FC)-Comp64 (0.1mpk)
R347(FC)-Comp64 (0.3mpk)
R347(FC)-Comp64 (1mpk)
108(FC)-Comp64 (0.1mpk)
108(FC)-Comp64 (0.3mpk)
108(FC)-Comp64 (1mpk)
Tumor Volume (mm3)
1800
1600
1400
1200
1000
800
600
400
200
0
10
20
30
40
50
60
70
80
90
100
110
ADC Tx
Days Post Implantation

FIG. 26B

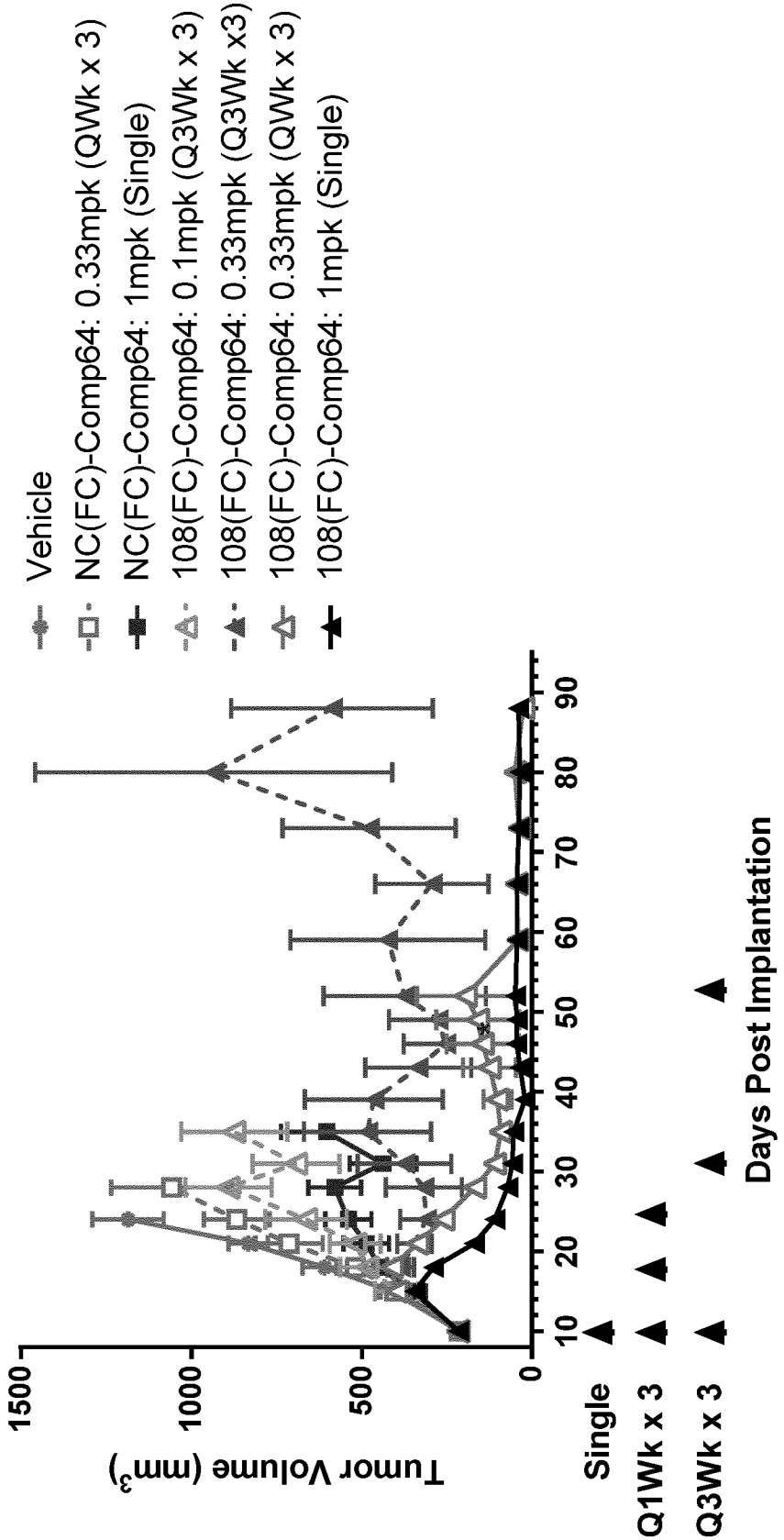

Vehicle
NC(FC)-Comp64: 0.33mpk (QWk x 3)
NC(FC)-Comp64: 1mpk (Single)
108(FC)-Comp64: 0.1mpk (Q3Wk x 3)
108(FC)-Comp64: 0.33mpk (Q3Wk x3)
108(FC)-Comp64: 0.33mpk (QWk x 3)
108(FC)-Comp64: 1mpk (Single)
Tumor Volume (mm3)
1500
1000
500
0
10
20
30
40
50
60
70
80
90
Days Post Implantation
Single
Q1Wk x 3
Q3Wk x 3

FIG. 27A

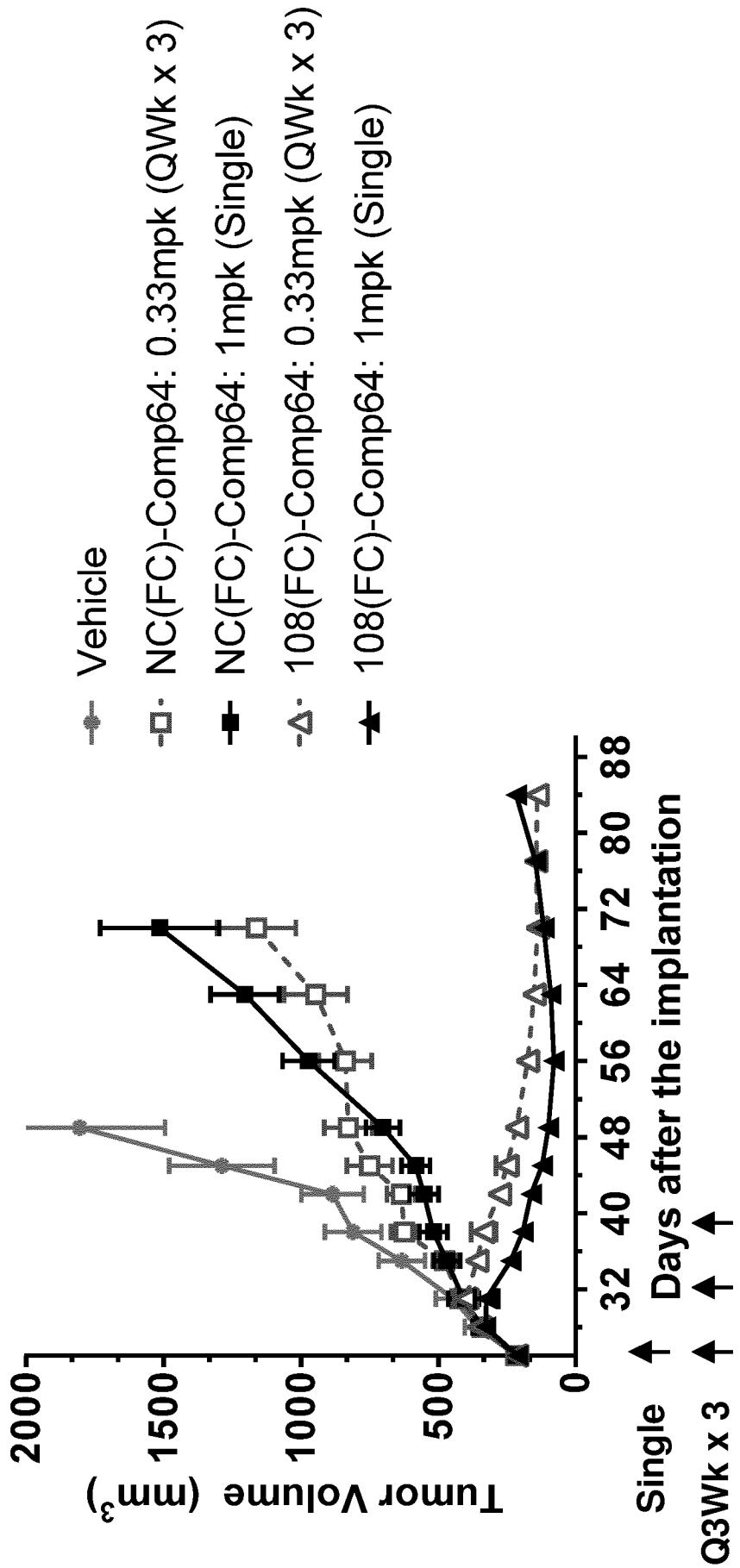

Vehicle
NC(FC)-Comp64: 0.33mpk (QWk x 3)
NC(FC)-Comp64: 1mpk (Single)
108(FC)-Comp64: 0.33mpk (QWk x 3)
108(FC)-Comp64: 1mpk (Single)
Tumor Volume (mm3)
2000
1500
1000
500
0
32 40 48 56 64 72 80 88
Days after the implantation
Single
Q3Wk x 3

FIG. 27B

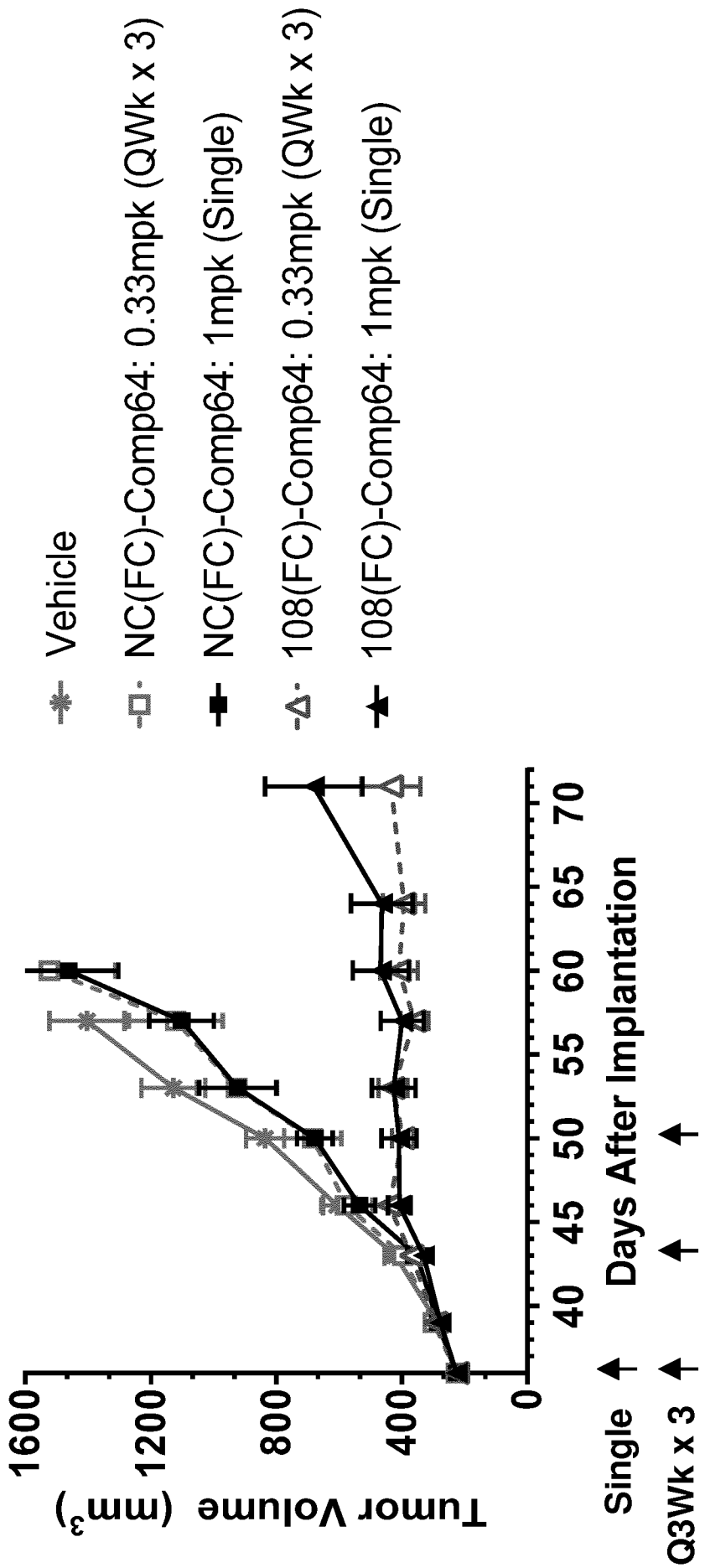
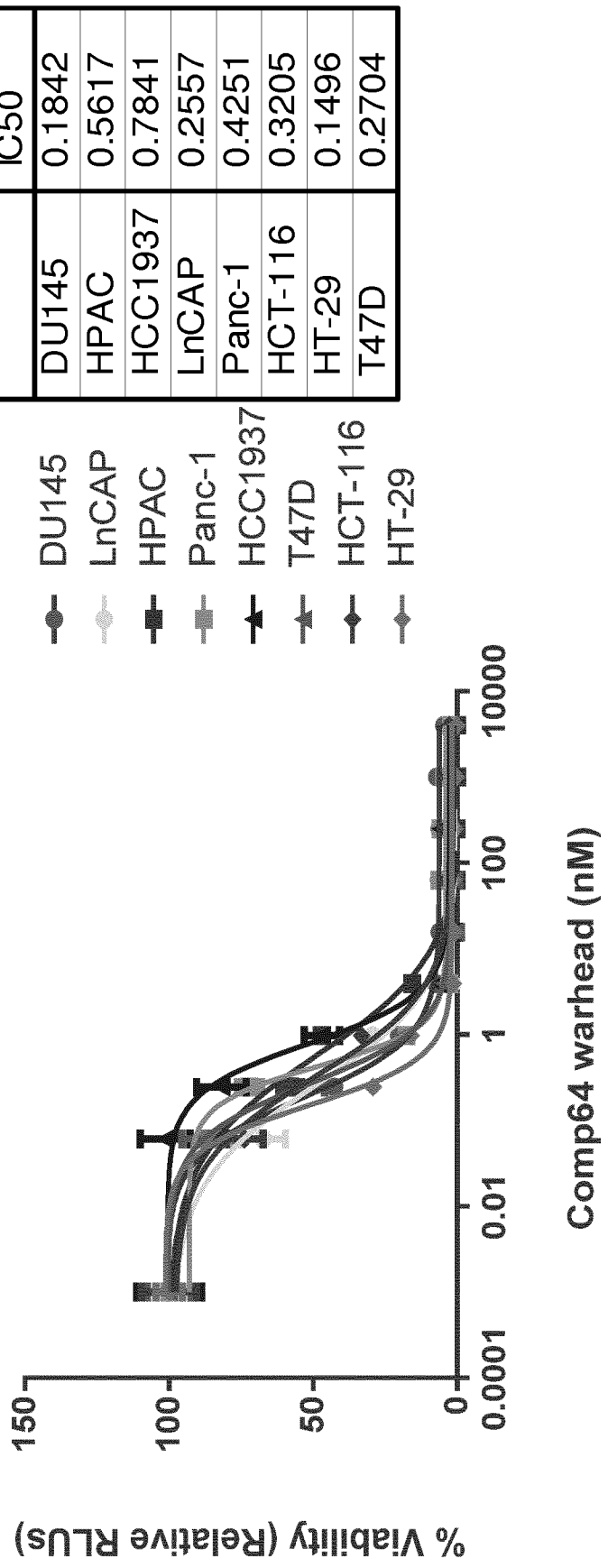

Vehicle
NC(FC)-Comp64: 0.33mpk (QWk x 3)
NC(FC)-Comp64: 1mpk (Single)
108(FC)-Comp64: 0.33mpk (QWk x 3)
108(FC)-Comp64: 1mpk (Single)
Tumor Volume (mm3)
1600
1200
800
400
0
40
45
50
55
60
65
70
Days After Implantation
Single
Q3Wk x 3

FIG. 28



	IC50
DU145	0.1842
HPAC	0.5617
HCC1937	0.7841
LnCAP	0.2557
Panc-1	0.4251
HCT-116	0.3205
HT-29	0.1496
T47D	0.2704

FIG. 29

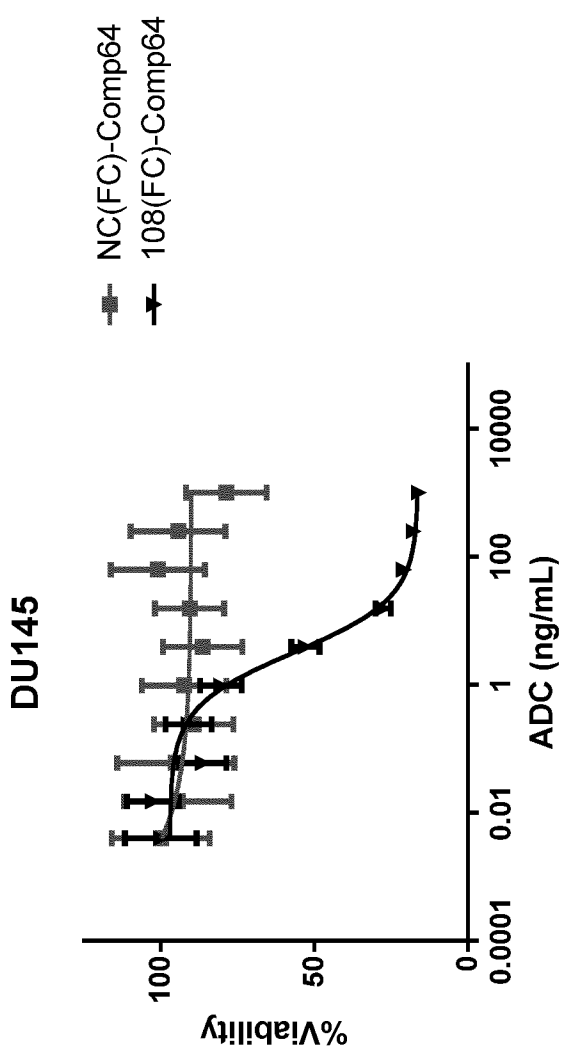
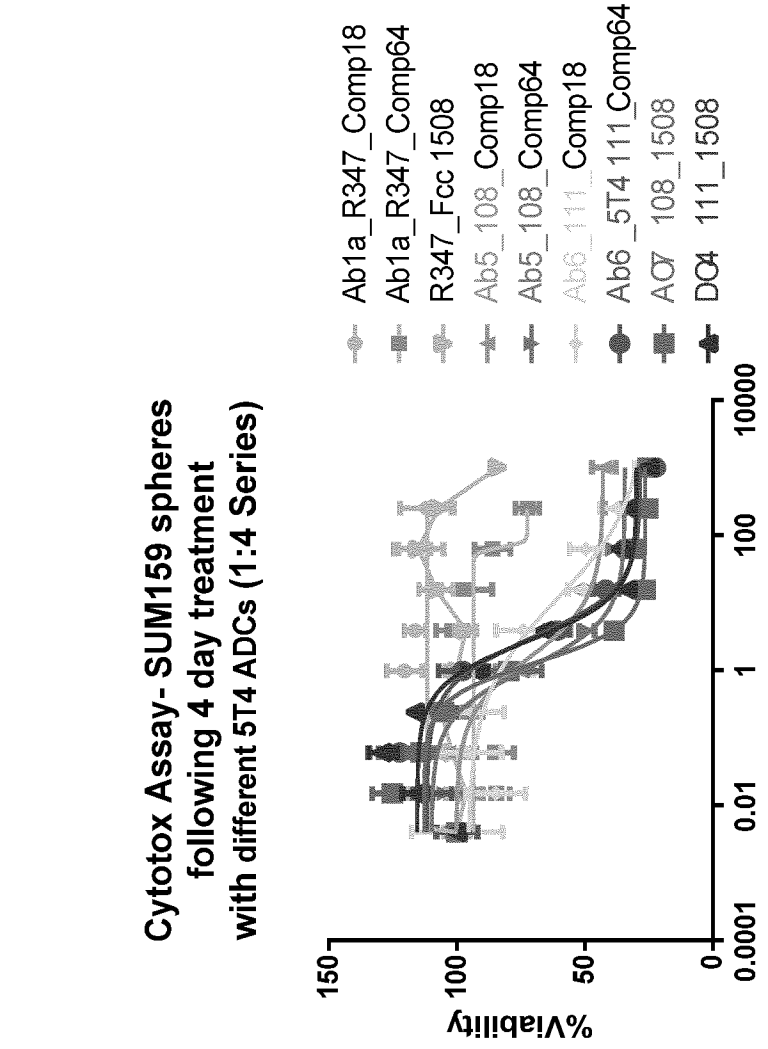
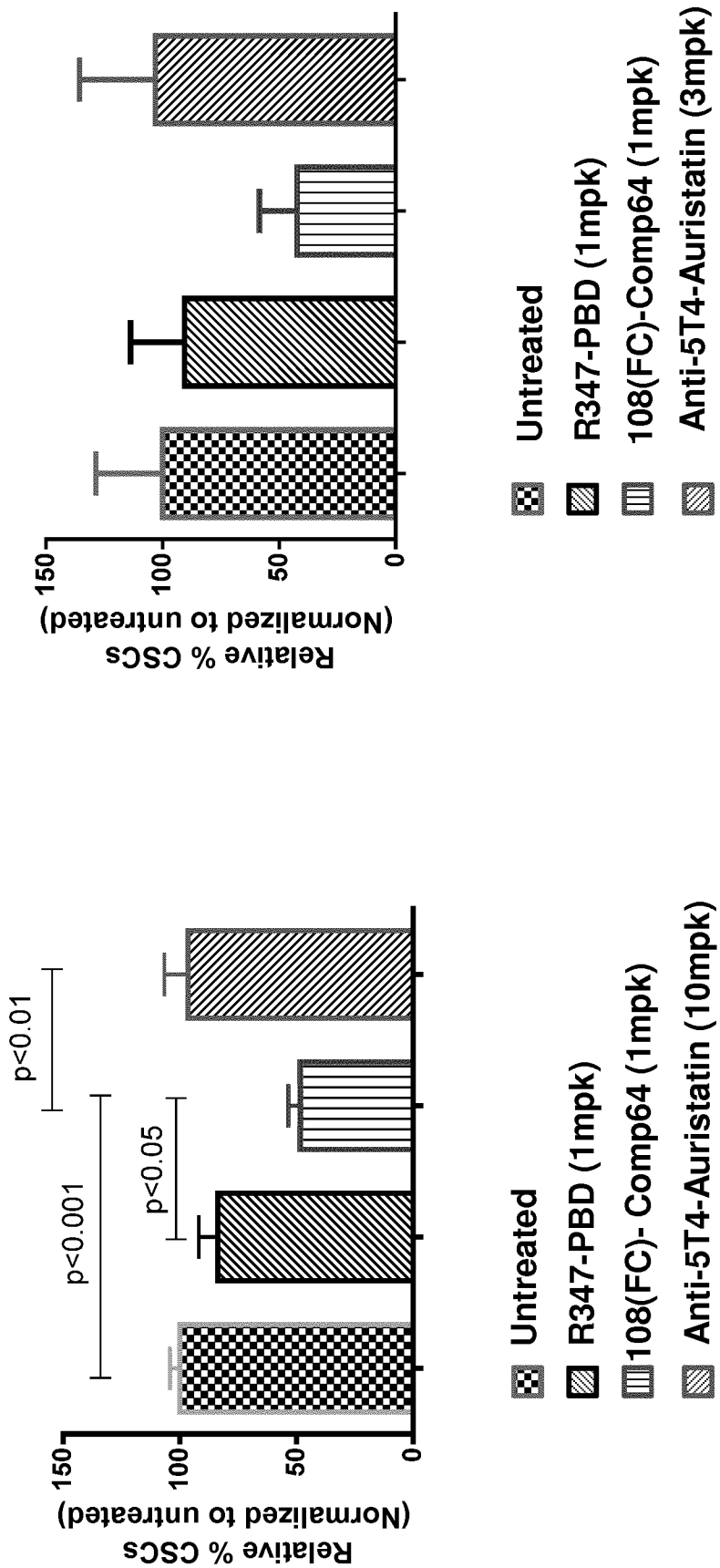

NC(FC)-Comp64
108(FC)-Comp64
DU145
%Viability
100
50
0
0.0001
0.01
1
100
10000
ADC (ng/mL)

FIG. 30

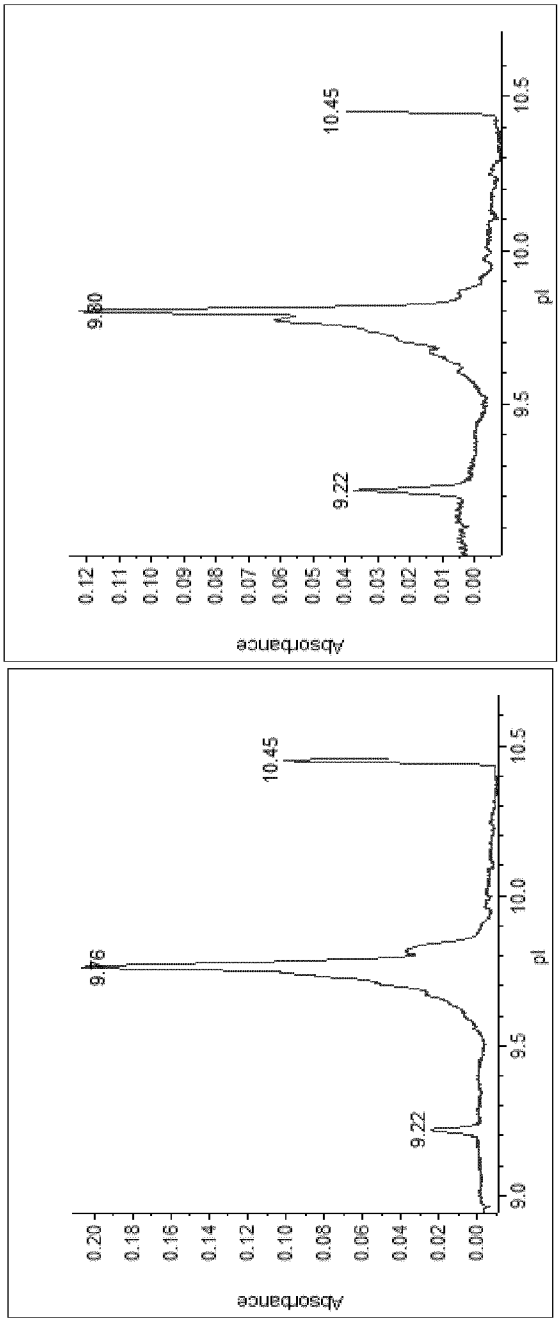


	Ab1a_R347_Comp18	Ab1a_R347_Comp64	Ab5_108_Comp18	Ab5_108_Comp64	Ab6_111_Comp18	Ab6_5T4 111_Comp64	AO7 108_1508	DO4 111_1508	R347_Fcc 1508
IC50	~ 0.003250	~ 65.78	1.554	1.079	11.55	3.094	1.282	2.677	

FIGS. 31A & 31B


Relative % CSCs
(Normalized to untreated)
150
100
50
0
p<0.001
p<0.05
p<0.01
Untreated
R347-PBD (1mpk)
108(FC)- Comp64 (1mpk)
Anti-5T4-Auristatin (10mpk)
Relative % CSCs
(Normalized to untreated)
150
100
50
0
Untreated
R347-PBD (1mpk)
108(FC)-Comp64 (1mpk)
Anti-5T4-Auristatin (3mpk)

FIGS. 32A & 32B


A
Unconjugated
B
Conjugated with 1508
Absorbance
pI
9.22
9.76
9.80
10.45
9.0
9.5
10.0
10.5

FIGS. 33A & 33B

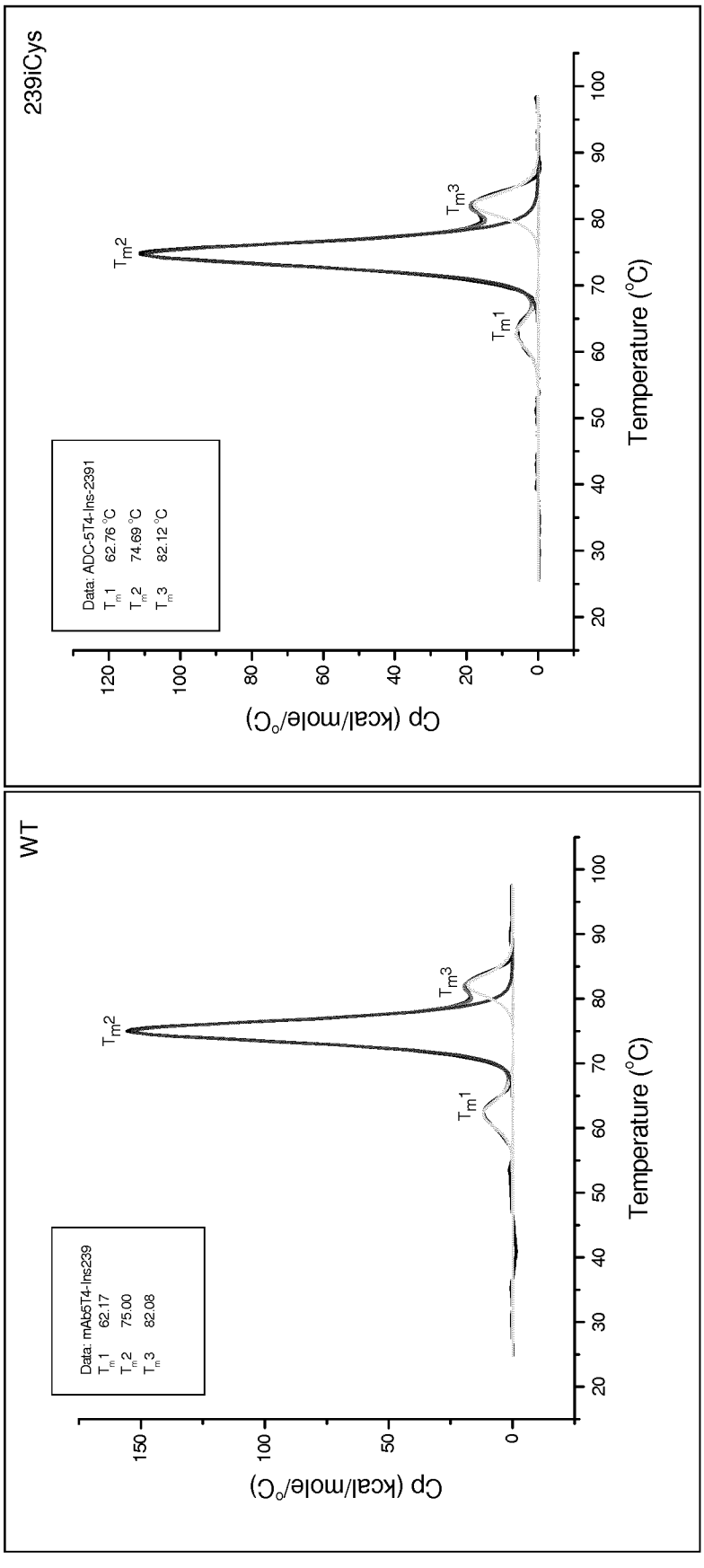

A
WT
Data: mAb5T4-Ins239
T_m1 62.17
T_m2 75.00
T_m3 82.08
Cp (kcal/mole/°C)
Temperature (°C)
B
239iCys
Data: ADC-5T4-Ins-2391
T_m1 62.76 °C
T_m2 74.69 °C
T_m3 82.12 °C
Cp (kcal/mole/°C)
Temperature (°C)

FIG. 34

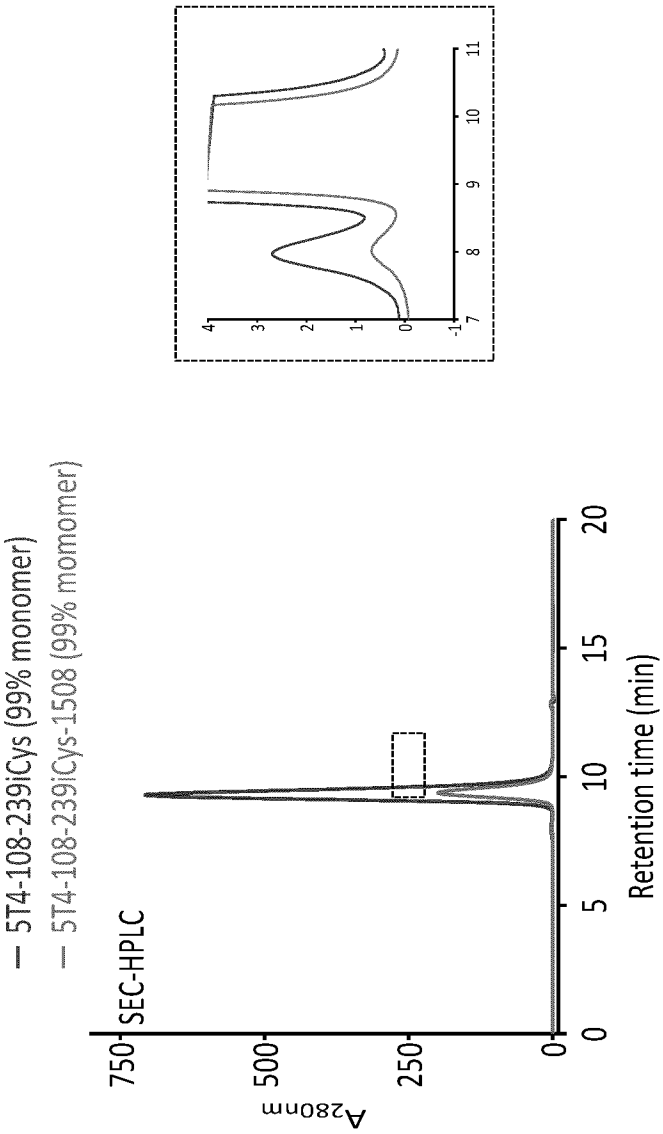

5T4-108-239iCys (99% monomer)
5T4-108-239iCys-1508 (99% momomer)
SEC-HPLC
A280nm
750
500
250
0
0
5
10
15
20
Retention time (min)
4
3
2
1
0
-1
7
8
9
10
11

FIG. 35

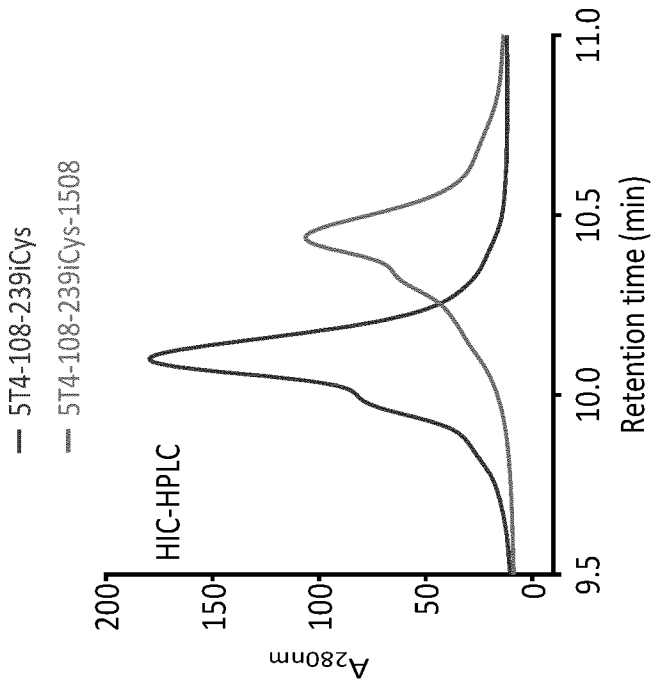

5T4-108-239iCys
5T4-108-239iCys-1508
HIC-HPLC
A280nm
200
150
100
50
0
9.5
10.0
10.5
11.0
Retention time (min)

FIG. 36

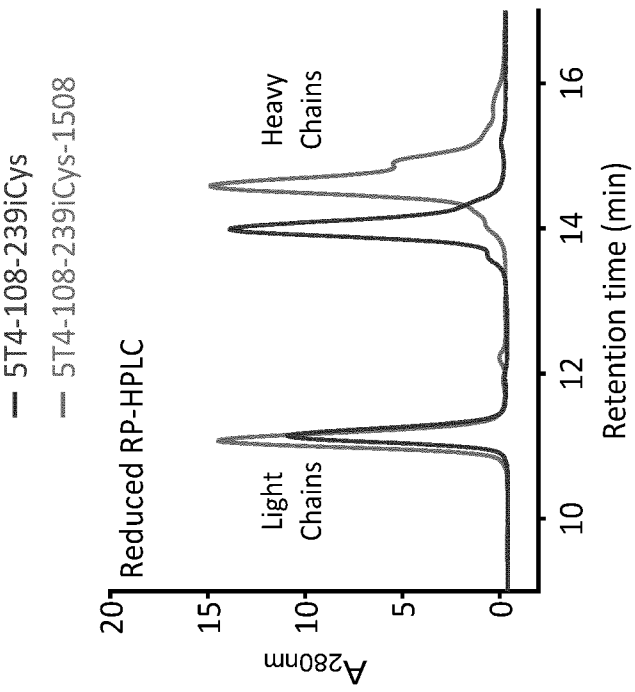

5T4-108-239iCys
5T4-108-239iCys-1508
Reduced RP-HPLC
Light Chains
Heavy Chains
A280nm
0
5
10
15
20
10
12
14
16
Retention time (min)

FIG. 37

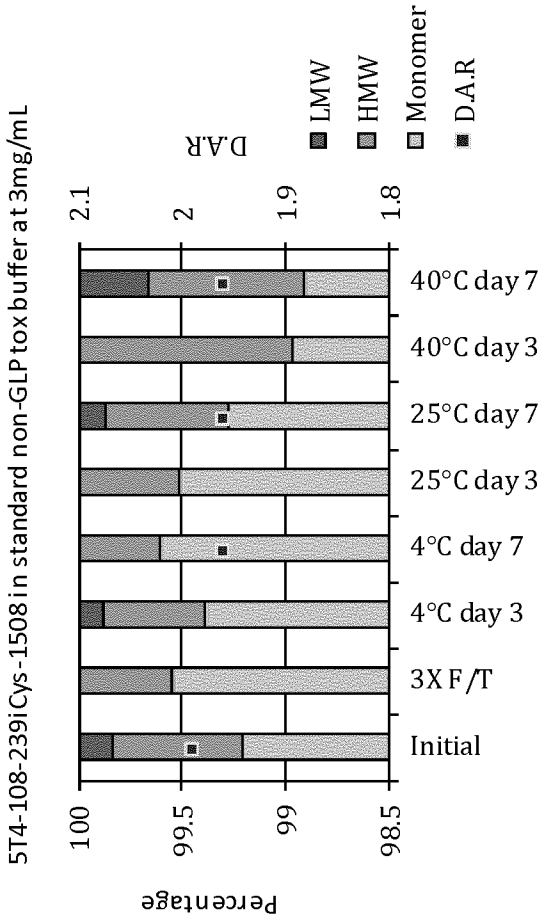
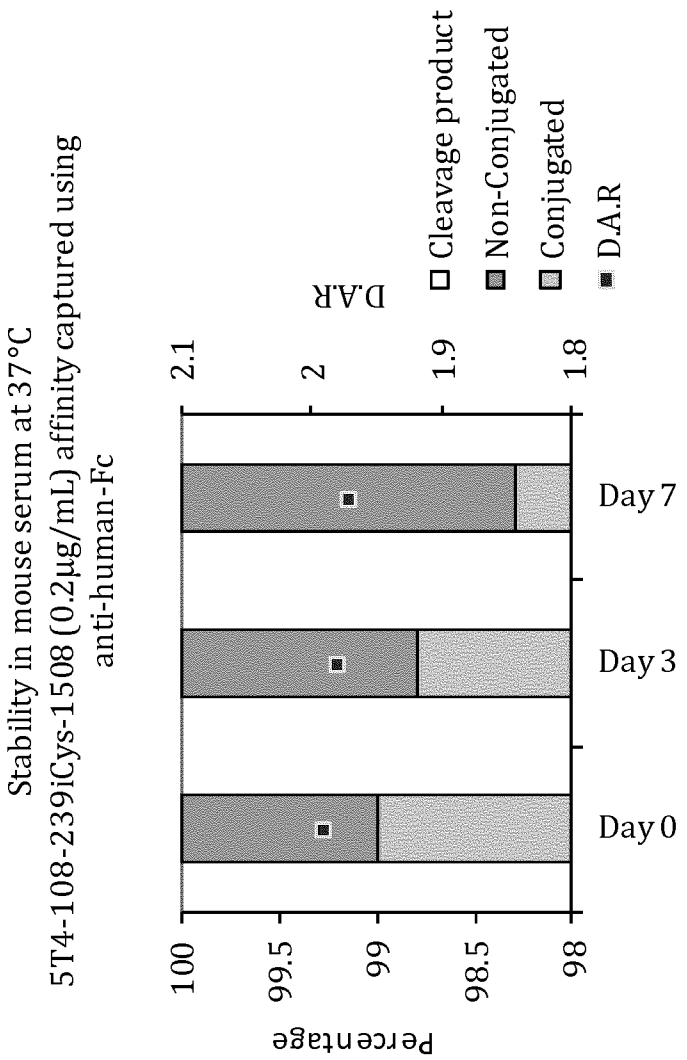

5T4-108-239iCys-1508 in standard non-GLP tox buffer at 3mg/mL
Percentage
100
99.5
99
98.5
D.A.R
2.1
2
1.9
1.8
LMW
HMW
Monomer
D.A.R
40°C day 7
40°C day 3
25°C day 7
25°C day 3
4°C day 7
4°C day 3
3X F/T
Initial

FIG. 38


Stability in mouse serum at 37°C
5T4-108-239iCys-1508 (0.2µg/mL) affinity captured using anti-human-Fc
Percentage
100
99.5
99
98.5
98
D.A.R
2.1
2
1.9
1.8
Day 0
Day 3
Day 7
Cleavage product
Non-Conjugated
Conjugated
D.A.R

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/057873

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/30 A61K47/48 A61P35/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	F. TIAN ET AL: "A general approach to site-specific antibody drug conjugates", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 5, 17 January 2014 (2014-01-17), pages 1766-1771, XP055122307, ISSN: 0027-8424, DOI: 10.1073/pnas.1321237111 the whole document -/--	1-175

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 June 2015	01/07/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Covone-van Hees, M

1

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& F. TIAN ET AL: "A general approach to site-specific antibody drug conjugates", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 5, 17 January 2014 (2014-01-17), pages 1766-1771, XP055101730, ISSN: 0027-8424, DOI: 10.1073/pnas.1321237111 the whole document	
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WOODS ANDREW M ET AL: "Characterization of the murine 5T4 oncofoetal antigen: A target for immunotherapy in cancer", BIOCHEMICAL JOURNAL, PORTLAND PRESS LTD, GB, vol. 366, no. 1, 15 August 2002 (2002-08-15), pages 353-365, XP002341798, ISSN: 0264-6021 the whole document	1-175
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E	WO 2015/054669 A1 (ASANA BIOSCIENCES LLC [US]) 16 April 2015 (2015-04-16) the whole document	1-175

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2015/057873

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [ ] forming part of the international application as filed:

      [ ] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [X] furnished subsequent to the international filing date for the purposes of international search only:

      [X] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/057873

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/057873

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