ANTI-HUMAN CACNG1 ANTIBODY-DRUG CONJUGATES AND USES THEREOF

TECHNICAL FIELD

[0001] The application relates to conjugates comprising an anti-hCACNG1 antibody (or antigen-binding molecules comprising an antigen-binding fragment of an anti-hCACNG1 antibody) and a therapeutic agent, which conjugates may be useful in treating diseases. The application further relates to methods of preparing and using conjugates comprising an anti-hCACNG1 antibody (or antigen-binding molecules comprising an antigen-binding fragment of an anti-hCACNG1 antibody) and a therapeutic agent.

SEQUENCE LISTING

[0002] A Sequence Listing in xml format entitled "250298_000513_SL.xml," which was created October 31, 2023, and is 337,817 bytes, is incorporated herein by reference in its entirety.

BACKGROUND

[0003] Skeletal muscle is the largest organ in the body, comprising ~40% of total body mass. Skeletal muscle is one of the three significant muscle tissues in the human body. Each skeletal muscle consists of thousands of muscle fibers wrapped together by connective tissue sheaths. The individual bundles of muscle fibers in a skeletal muscle are known as fasciculi. The outermost connective tissue sheath surrounding the entire muscle is known as epimysium. The connective tissue sheath covering each fasciculus is known as perimysium, and the innermost sheath surrounding individual muscle fiber is known as endomysium. Each muscle fiber is comprised of a number of myofibrils containing multiple myofilaments.

[0004] When bundled together, all the myofibrils get arranged in a unique striated pattern forming sarcomeres which are the fundamental contractile unit of a skeletal muscle. The two most significant myofilaments are actin and myosin filaments arranged distinctively to form various bands on the skeletal muscle.

[0005] The primary functions of the skeletal muscle take place via its intrinsic excitation-contraction coupling process. As the muscle is attached to the bone tendons, the contraction of the muscle leads to movement of that bone that allows for the performance of specific movements. The skeletal muscle also provides structural support and helps in maintaining the posture of the body. The skeletal muscle also acts as a storage source for amino acids that can be used by different

organs of the body for synthesizing organ-specific proteins. The skeletal muscle also acts as a site of glucose disposal in the form of muscle glycogen. The skeletal muscle also plays a central role in maintaining thermostasis and acts as an energy source during starvation. Thus, skeletal muscle plays key roles in locomotion, thermoregulation, and in controlling whole body metabolism.

[0006] In many muscle diseases as well as during normal aging, the size and function of skeletal muscle tissue is reduced, resulting in impaired functional mobility; and in the case of severe muscle diseases, long-term disability and early mortality.

[0007] Treatments for muscle wasting and genetic muscle diseases typically consist of broad-acting therapies, such as testosterone therapy for muscle wasting, glucocorticoids for muscular dystrophies, etc. Untargeted delivery of these therapies reduces efficiency of specific muscle uptake, while also causing significant detrimental off-target effects on other organs.

[0008] There is a need in the art for new anti-human antibodies and antibody-drug conjugate therapeutics, capable of binding a muscle-specific marker and effecting the internalization by muscle cells of a therapeutic payload.

SUMMARY

[0009] Described herein are antibody drug conjugates comprising an antibody, or antigen-binding fragment thereof, that bind human CACNG1. Described herein are antibody-drug conjugates comprising an anti-hCACNG1 antibody, antigen-binding fragment thereof, as described herein and a payload (e.g., a therapeutic payload, e.g., a therapeutic small molecule). In some embodiments, the (a) anti-hCACNG1 antibody, antigen-binding fragment and/or multispecific binding protein and (b) the payload are covalently attached via a linker, as discussed herein. In various embodiments, the anti-hCACNG1 antibody or antigen-binding fragment can be any of the anti- CACNG1 antibodies or fragments described herein.

[0010] In one aspect, the present disclosure provides an antibody-drug conjugate comprising an antigen-binding protein that specifically binds to Human Calcium Voltage Gated Channel Auxiliary Subunit Gamma 1 (hCACNG1), wherein the antigen-binding protein is conjugated directly or via a linker to at least one therapeutic agent, or a pharmaceutically acceptable salt thereof.

[0011] In one embodiment, the antibody-drug conjugate has a structure according to Formula (I):

$A - [L - P]_y$ (I),

wherein A is the antigen-binding protein;

L is absent or a linker;

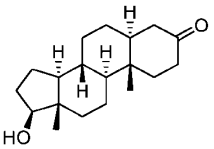
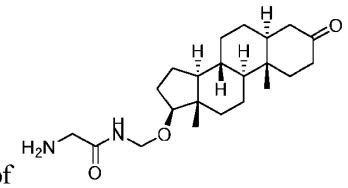
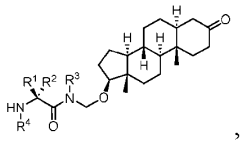
P is a therapeutic agent, and

y is an integer from 1 to 8.

[0012] In one embodiment, the anti-hCACNG1 antibody or antigen-binding fragment thereof comprises a heavy chain variable region (HCVR or VH) and/or a light chain variable region (LCVR or VL).

[0013] In one embodiment, the anti-hCACNG1 antibody or antigen-binding fragment thereof comprises a heavy chain variable region (HCVR or VH) and/or a light chain variable region (LCVR or VL).

[0014] In one embodiment, the anti-hCACNG1 antibody or antigen-binding fragment thereof comprises an HCVR/LCVR amino acid sequence pair having at least 90% sequence identity to an HCVR/LCVR amino acid sequence pair selected from the group consisting of SEQ ID NOs: 2/10, SEQ ID NOs: 18/26, SEQ ID NOs: 34/42, SEQ ID NOs: 50/58, SEQ ID NOs: 66/74, SEQ ID NOs: 82/90, SEQ ID NOs: 98/106, SEQ ID NOs: 114/122, SEQ ID NOs: 130/138, SEQ ID NOs: 146/154,SEQ ID NOs: 162/170, SEQ ID NOs: 178/186, SEQ ID NOs: 294/301, and SEQ ID NOs: 314/322.

[0015] In one embodiment, the therapeutic agent is dihydrotestosterone (DHT), or a prodrug or derivative thereof. In one embodiment, the therapeutic agent is selected from the group consisting of DHT having the structure  and proDHT having the structure selected from the group consisting of  and  ,

wherein R_1 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

R_2 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

R_3 is selected from the group consisting of H and a C_{1-3} alkyl, and

R_4 is selected from the group consisting of H and a C_{1-3} alkyl.

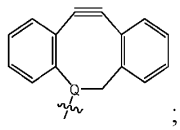
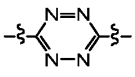
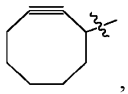
[0016] In one embodiment, the therapeutic agent is conjugated to the antigen-binding protein via the linker L.

[0017] In one embodiment, n is 2 or 4.

[0018] In one embodiment, the linker has the structure:

-L1-B-L2- , wherein:

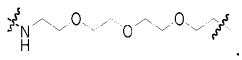
L1 is a first linker unit covalently attached to the antigen-binding protein;

B is absent or a unit comprising at least one adduct of group B', where the group B' is selected from -N_3,  ;  ; and  , where Q is C or N;

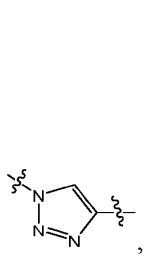
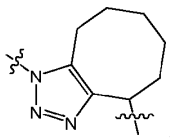
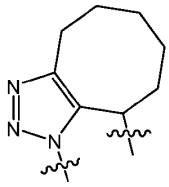
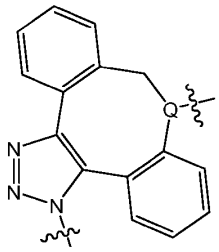
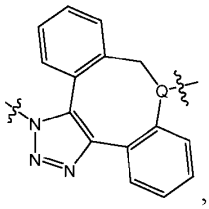
L2 is absent or a second linker unit covalently attached to the unit B via at least one group B", wherein the group B' and the group B" form the at least one adduct,

provided that when B is absent, L2 is also absent and L1 is covalently attached to the therapeutic agent, and when L2 is present, L2 is covalently attached to the therapeutic agent.

[0019] In one embodiment, the L1 comprises C_{1-6} alkyl, phenyl, -NH-, -C(O)-, -$(CH_2)_u$-NH-C(O)-, -$(CH_2)_u$-C(O)-NH-, -$(CH_2$-CH_2-$O)_v$-, -$(CH_2)_u$-$(O$-CH_2-$CH_2)_v$-C(O)-NH-, a peptide unit comprising from 2 to 4 amino acids, or combinations thereof, each of which may be optionally substituted with one or more of -S-, -$S(O_2)$-, -C(O)-, -$C(O_2)$-; and CO_2H, wherein subscripts u and v are independently an integer from 1 to 8.

[0020] In one embodiment, the L1 is  .

[0021] In one embodiment, the B has a structure selected from the group consisting of:

 ,  ,  ,  , and  ,

wherein Q is C or N.

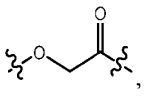
[0022] In one embodiment, the L2 has a structure:

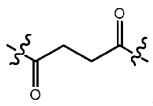
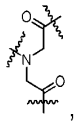
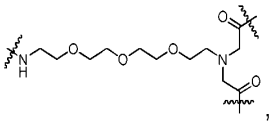
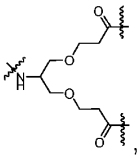
-SP1-AA-SP2- (L2), wherein:

SP1 is absent or a first spacer unit;

AA is absent or a peptide unit comprising from 2 to 4 amino acids;

SP2 is absent or a second spacer unit covalently attached to the therapeutic agent.

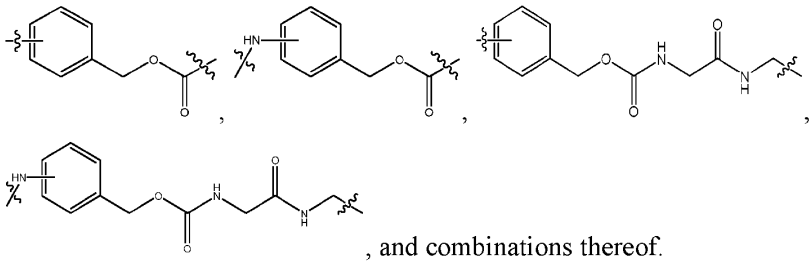
[0023] In one embodiment, the SP1 is absent or selected from the group consisting of  ,

 ,  ,  ,  , $C_{1\text{-}6}$ alkyl, -$(CH_2$-CH_2-$O)_v$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O), -O-CH_2-C(O)-NH, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-NH, -NH-, -C(O)-, -NH-C(O)-, -NH-$(CH_2)_u$-, -NH-$(CH_2)_u$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-, -NH-$(CH_2$-CH_2-$O)_v$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-, -$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-C(O)-NH-, or combinations thereof; wherein subscripts u and v are independently an integer from 1 to 8.

[0024] In one embodiment, the AA is a peptide unit comprising from 2 to 4 amino acids selected from glycine, valine, phenylalanine, proline, glutamic acid, lysine, N,N-dipropyl lysine, phenylalanine, and citrulline, and combinations thereof.

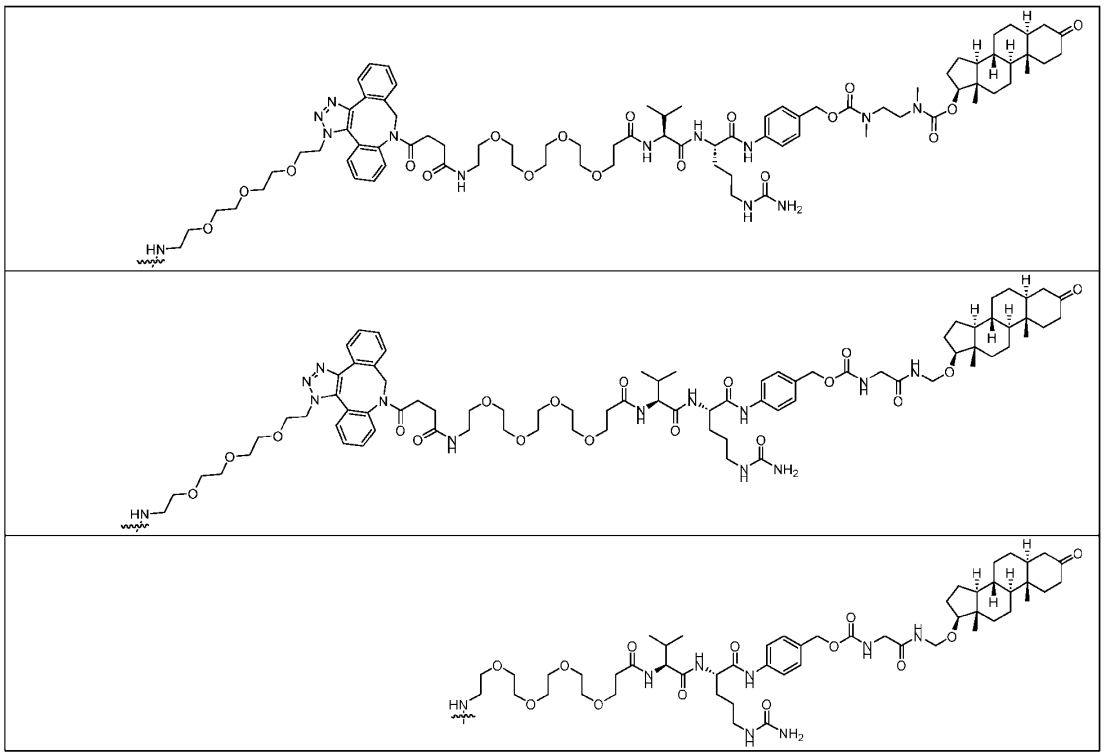
[0025] In one embodiment, the AA is valine-citrulline, valine-alanine, valine-lysine, valine-N,N-dipropyl lysine, phenylalanine-lysine, glycine-glycine-glycine (GGG), glycine-glycine-glycine-glycine (GGGG (SEQ ID NO: 289)), glycine-glycine-phenylalanine (GGF), glycine-glycine-phenylalanine-glycine (GGFG (SEQ ID NO: 290)), L-glutamic acid-valine-citrulline (^{L}EVC), and D-glutamic acid-valine-citrulline (^{D}EVC).

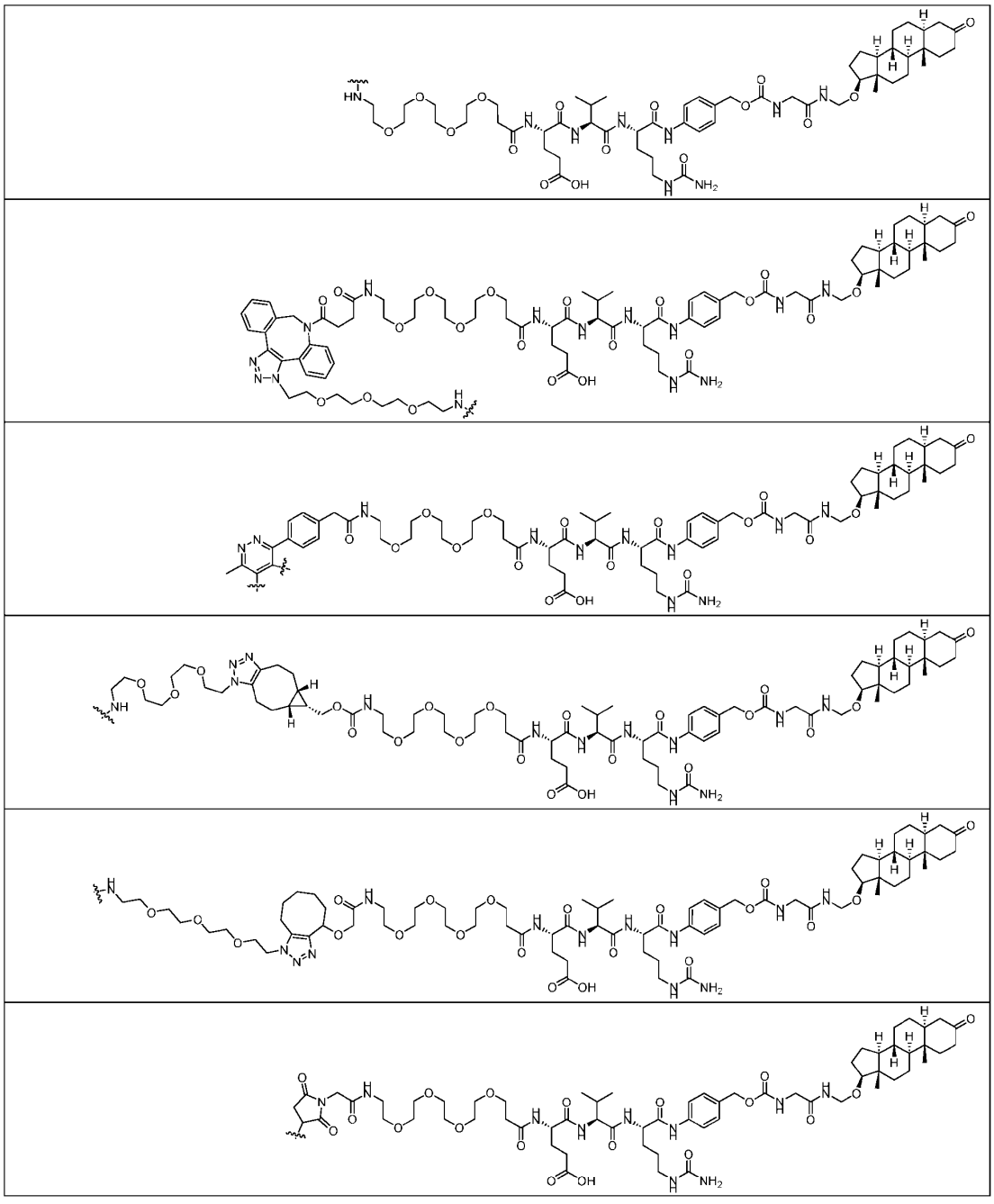
[0026] In one embodiment, the SP2 is absent or selected from the group consisting of

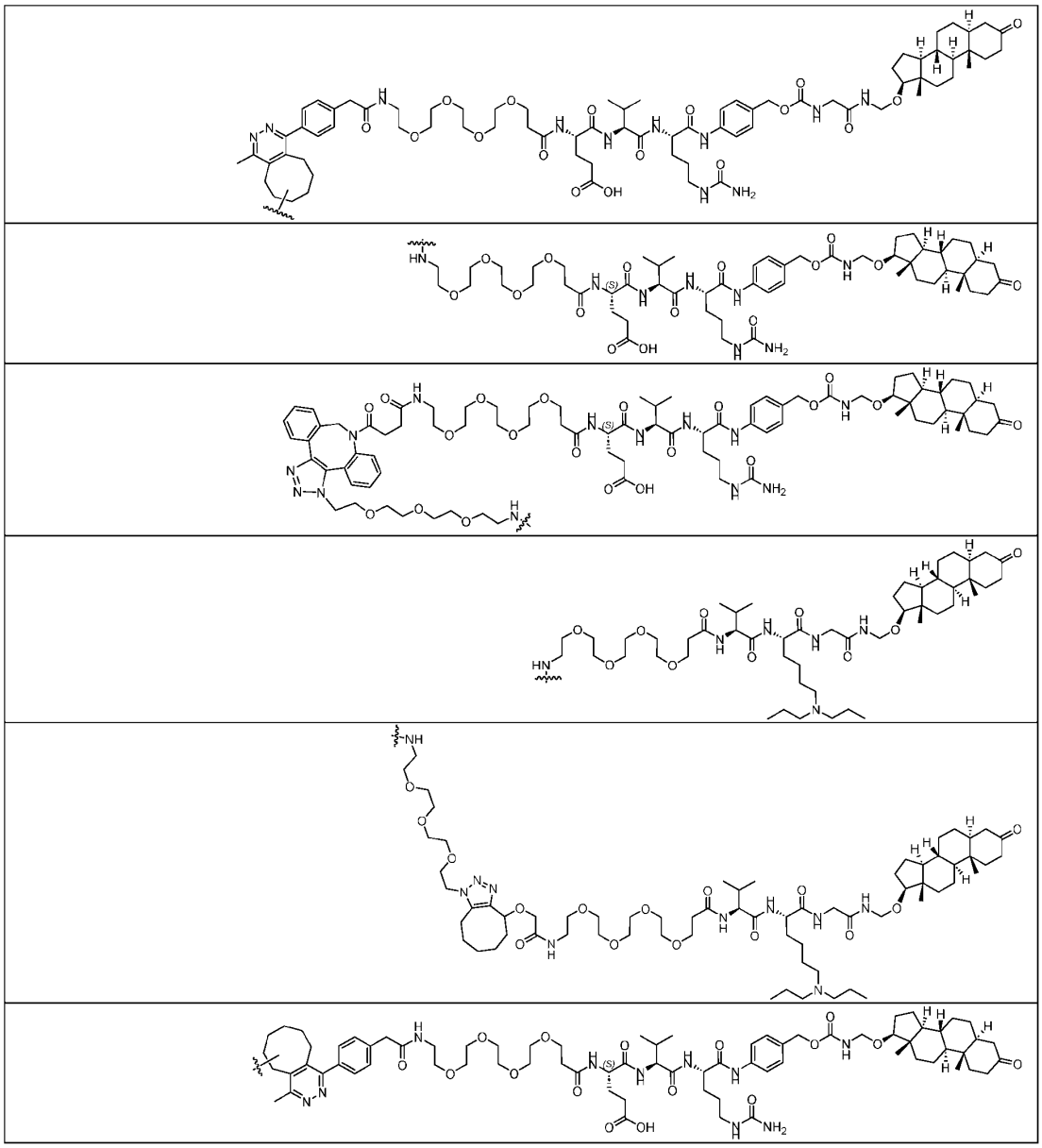


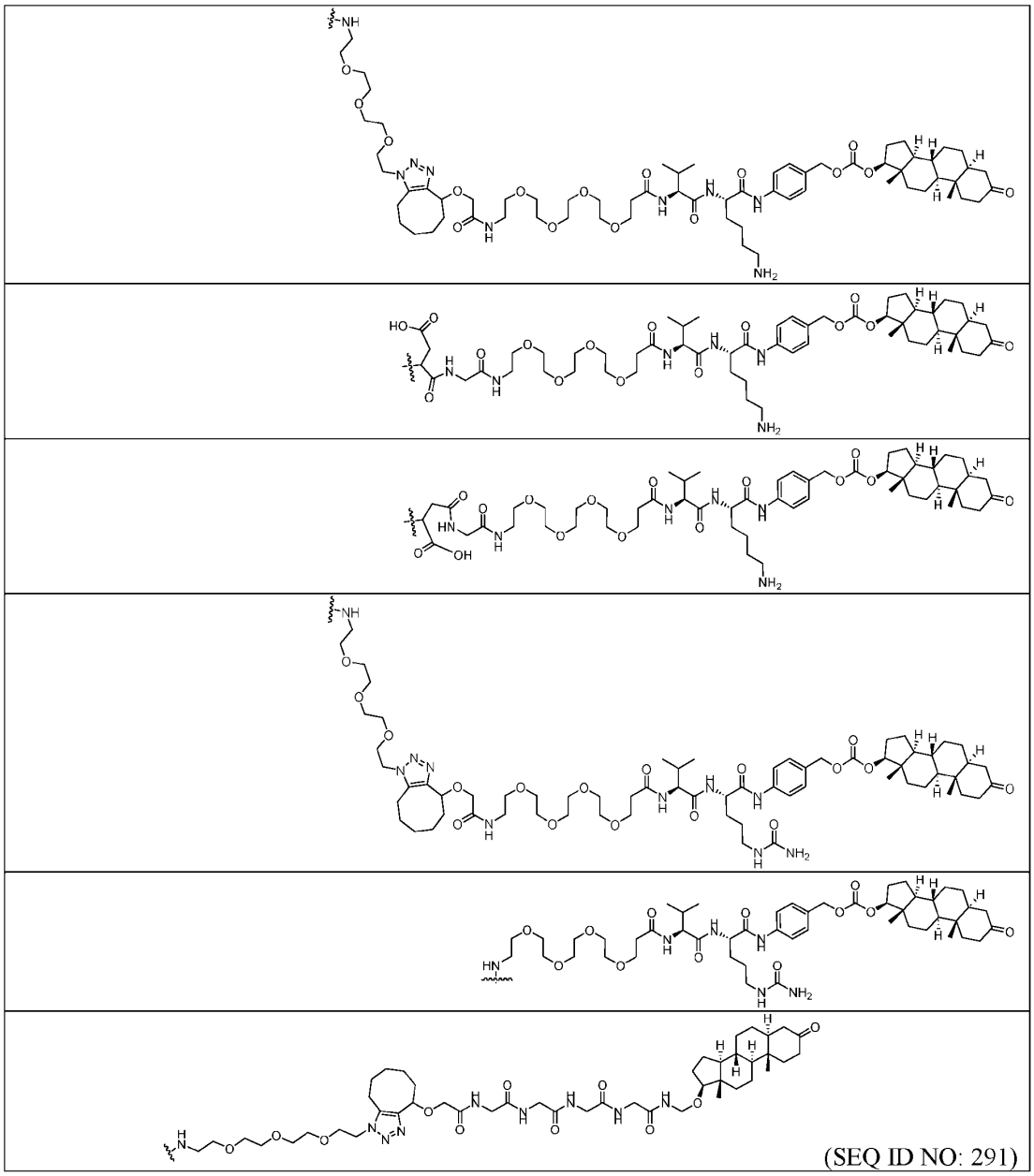
, and combinations thereof.

[0027] In one embodiment, the linker-therapeutic agent (L-P) has a structure selected from the group consisting of:

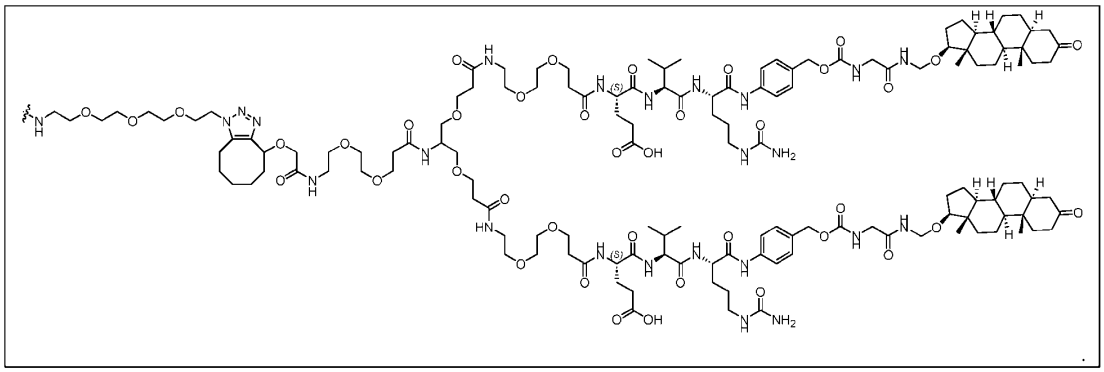








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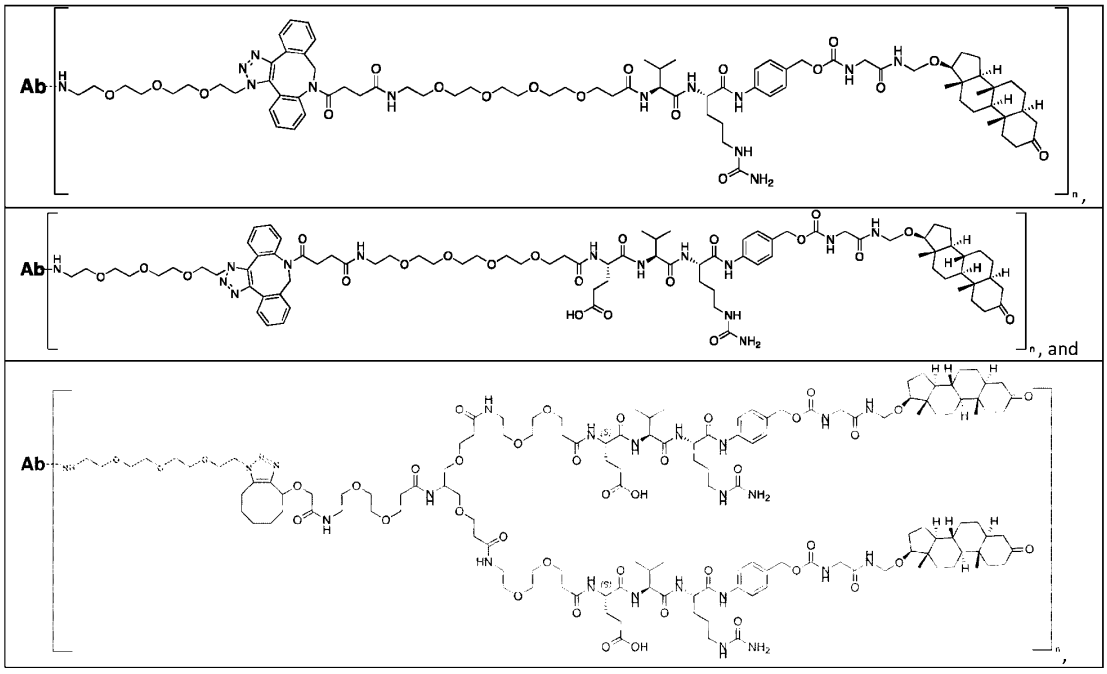


[0028] In one embodiment, the linker is attached to the side chain of a glutamine residue of the the antigen-binding protein.

[0029] In one embodiment, the glutamine residue is naturally present in a CH2 or CH3 domain of the antigen-binding protein.

[0030] In one embodiment, the glutamine residue is introduced to the antigen-binding protein by modifying one or more amino acids.

[0031] In one embodiment, the antibody-drug conjugate has a structure selected from the group consisting of:



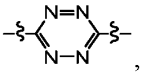
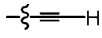
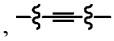
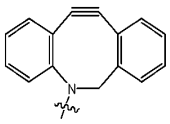
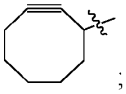
wherein Ab is the antigen-binding protein and n is an integer from 1 to 4.

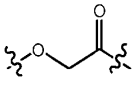
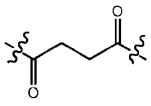
[0032] In another aspect, the present disclosure provides a compound according to Formula (L2-P) or (L2'-P):

B"-SP1-AA-SP2-P (L2-P),

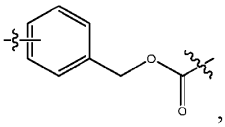
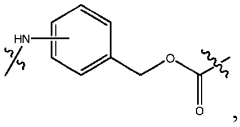
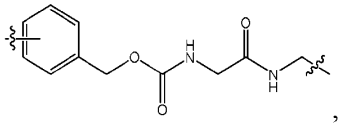
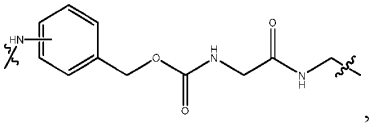
H_2N-SP1-AA-SP2-P)$_p$ (L2'-P),

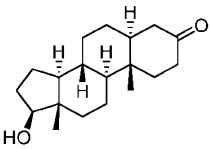
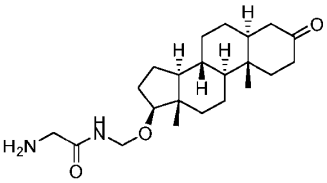
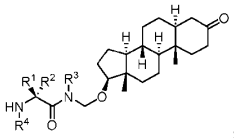
or a pharmaceutically acceptable salt thereof, wherein:

B" is selected from the group consisting of -N_3,  ,  ,  ,  and  ;

SP1 is absent or a first spacer unit selected from the group consisting of  and  ; a $C_{1\text{-}6}$ alkyl, -$(CH_2$-CH_2-$O)_v$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O), -O-CH_2-C(O)-NH, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-NH, -NH-, -C(O)-, -NH-C(O)-, -NH-$(CH_2)_u$-, -NH-$(CH_2)_u$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-, -NH-$(CH_2$-CH_2-$O)_v$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-, -$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-C(O)-NH-, or combinations thereof; wherein subscripts u and v are independently an integer from 1 to 8;

AA is absent or a peptide unit comprising from 2 to 4 amino acids;

SP2 is absent or a second spacer unit selected from the group consisting of  ,  ,  ,  , and combinations thereof, and

P is a therapeutic agent selected from the group consisting of DHT having the structure  and proDHT having the structure selected from the group consisting of  and  ,

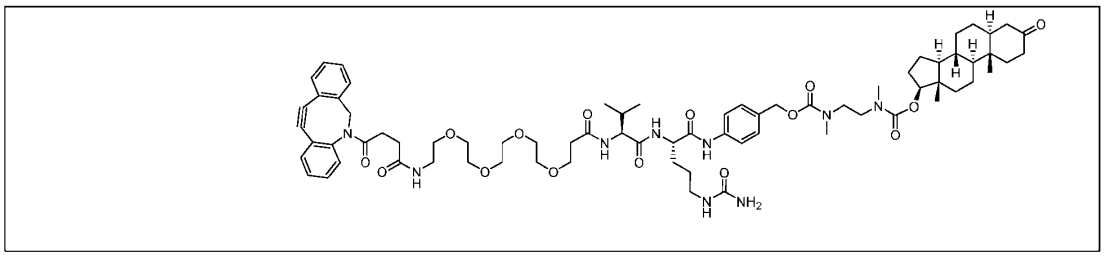
wherein R_1 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

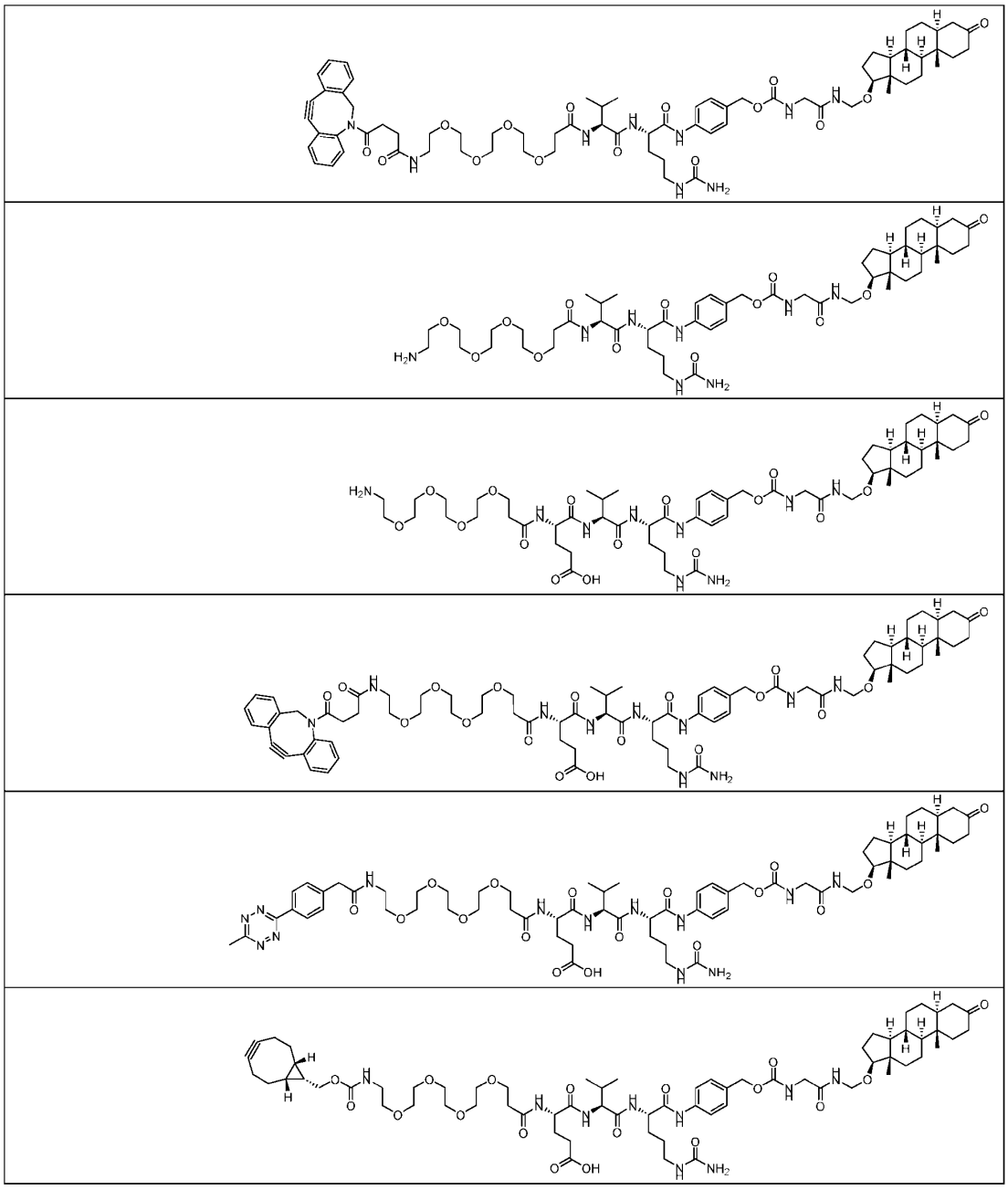
R_2 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

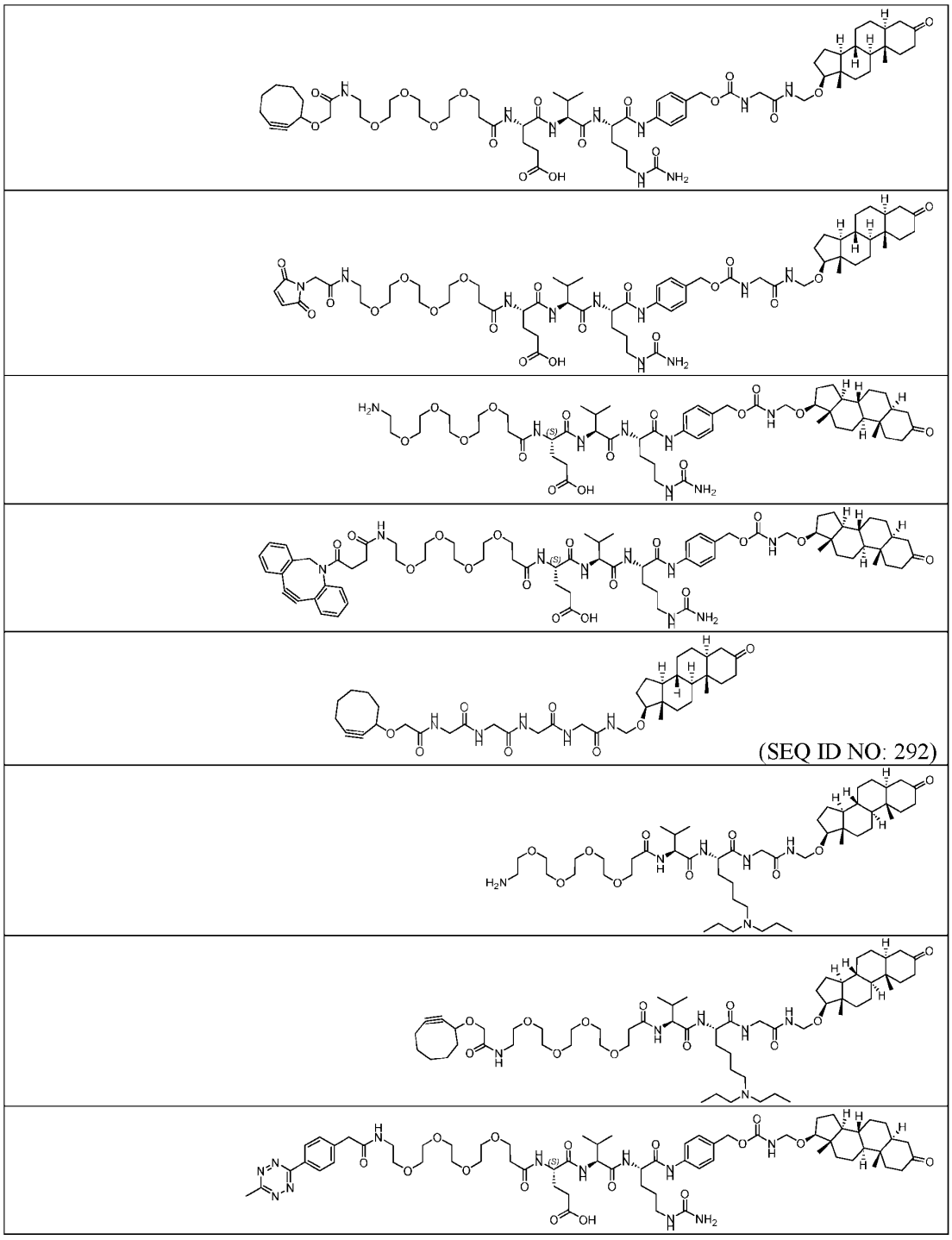
R_3 is selected from the group consisting of H and a C_{1-3} alkyl, and

R_4 is selected from the group consisting of H and a C_{1-3} alkyl.

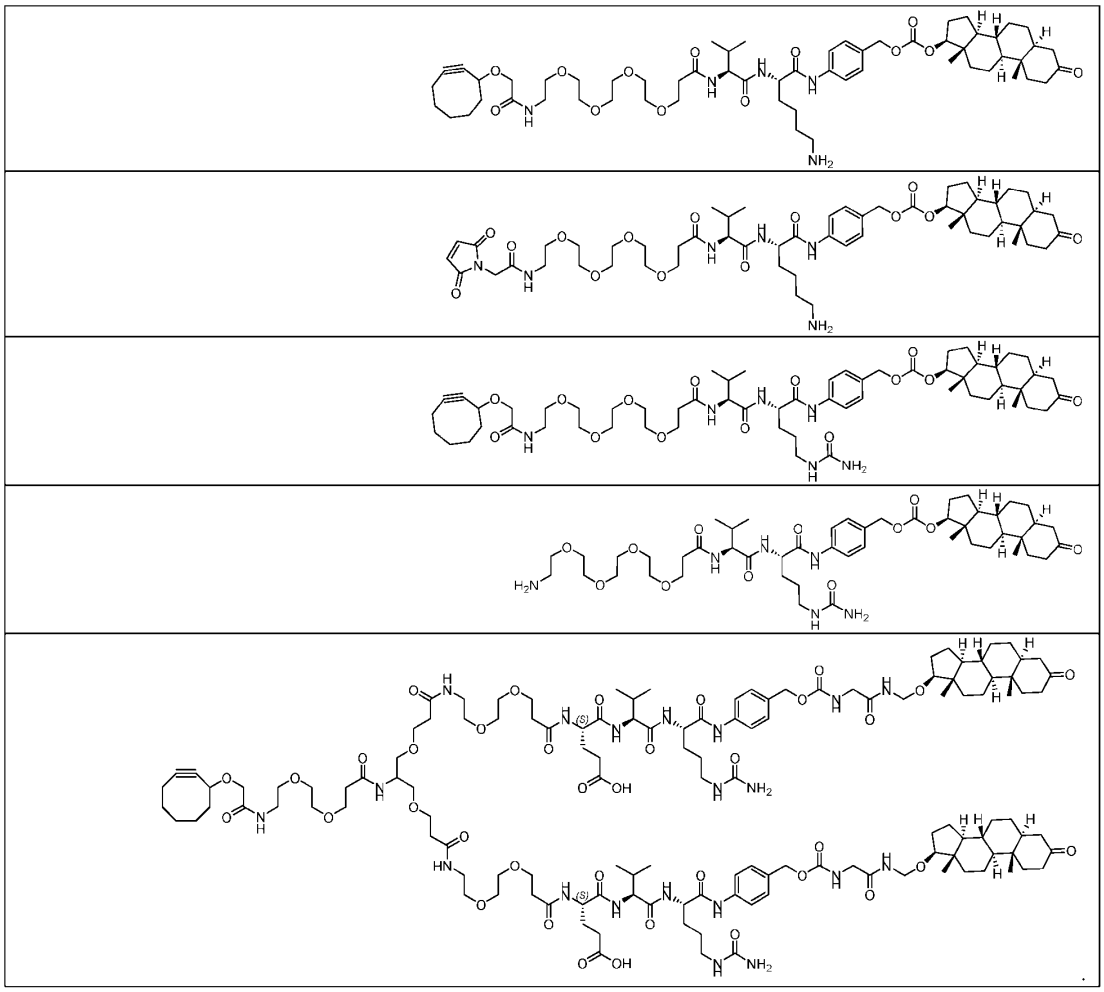
[0033] In one embodiment, the compound has a structure selected from the group consisting of:



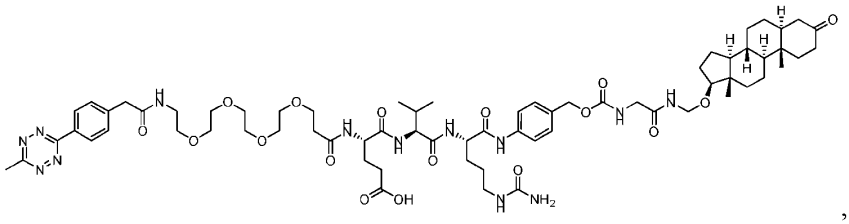




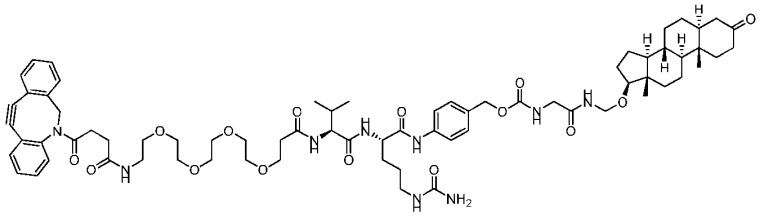
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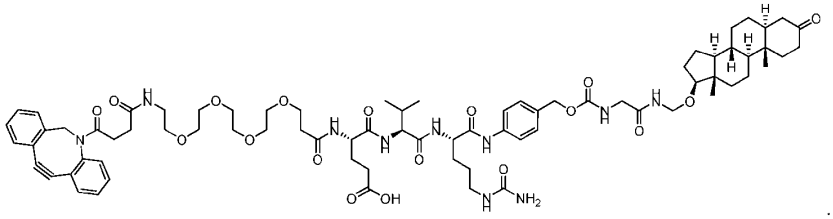
[0034] In yet another aspect, the present disclosure provides an antibody-drug conjugate or a pharmaceutically acceptable salt thereof comprising an antigen-binding protein that specifically binds to Human Calcium Voltage Gated Channel Auxiliary Subunit Gamma 1 (hCACNG1) conjugated to the linker-payload selected from the group consisting of



,



, and



.

[0035] In yet another aspect, the present disclosure provides a composition comprising a population of the antibody-drug conjugates of any of the above embodiments having a drug-antibody ratio (DAR) of about 0.5 to about 8.0.

[0036] In one embodiment, the composition has a DAR of about 1.0 to about 2.5

[0037] In one embodiment, the composition has a DAR of about 2.0.

[0038] In one embodiment, the composition has a DAR of about 3.0 to about 4.5

[0039] In one embodiment, the composition has a DAR of about 4.0.

[0040] In one embodiment, the present disclosure provides a pharmaceutical composition comprising the antibody-drug conjugate of any one the above embodiments and a diluent, a carrier, and/or an excipient.

[0041] In another aspect, the present disclosure provides a pharmaceutical dosage form comprising the antibody-drug conjugate of any of the above embodiments or the pharmaceutical composition of any of the above embodiments.

[0042] In yet another aspect, the present disclosure provides a method of treating a condition in a subject in need thereof, wherein the method comprises administering to the subject the antibody-drug conjugate of any of the above embodiments, the pharmaceutical composition of any of the above embodiments, or the pharmaceutical dosage form of any of the above embodiments.

[0043] In one embodiment, the condition is selected from the group consisting of muscle wasting and genetic muscle diseases.

[0044] In one embodiment, the condition is selected from the group consisting of adult spinal muscular atrophy, amyotrophic lateral sclerosis (ALS), anoctaminopathy, autoimmune neuropathy, Becker muscular dystrophy, Bethlem myopathy, calapainopathy, caveolinopathy, central core disease, Charcot-Marie-Tooth disease (CMT), congenital fibre type disproportination (CFTD), congenital muscular dystrophy, congenital myasthenic syndrome (CMS), congenital myopathy, congenital myotonic dystrophy, dermatomyositis (DM), disorders of the neuromuscular junction, distal myopathy with rimmed vacuoles (DMRV), Duchenne muscular dystrophy (DMD), Emery-Dreifuss muscular dystrophy, facioscapulohumeral muscular dystrophy (FSHD), facioscapuloperoneal muscular dystrophy (FSPD), fibrodysplasia ossificans progressiva (FOP), glycogen storage disease type V (GSD V), GNE myopathy (GNEM), hereditary inclusion body myopathy (HIBM), hereditary inclusion body myopathy type 2 (HIBM 2), hereditary motor and sensory neuropathy (HMSN), hereditary neuropathies, inclusion body myositis (IBM), infantile progressive spinal muscular atrophy, inflammatory neuropathy, infectious myelitis, intermediate spinal muscular atrophy, juvenile dermatomyositis, juvenile spinal muscular atrophy, Landouzy-Dejerine, limb girdle muscular dystrophies (LGMDs), McArdle disease, merosin-deficient congenital muscular dystrophy, metabolic myopathy, minicore myopathy, multicor myopathy, mitochondrial myopathy, muscular dystrophy, myasthenia gravis, myofibrillar myopathy, myositis, myositis ossificans progressiva (MOP), myotonic dystrophy, myotubular and other centronuclear myopathy, nemaline myopathy, Nonaka myopathy, oculopharyngeal muscular dystrophy (OPMD), periodic paralysis, polymyositis (PM) and dermatomyositis (DM), quadriceps-sparing myopathy (QSM), sarcoglycanopathy, sarcopenia, SEPN1-related myopathy, spinal muscular atrophy (SMA), Steinert's disease, Ullrich congenital muscular dystrophy, VCP disease, and age-related progressive loss of muscle mass and strength.

[0045] In one embodiment, the subject is an adult. In one embodiment, the subject is over 65 years of age.

[0046] In one embodiment, the subject is under 18 years of age.

[0047] In another aspect, the present disclosure provides a method of selectively delivering a compound into a cell, wherein the compound is the antibody-drug conjugate of any of the above embodiments.

[0048] In another aspect, the present disclosure provides a method of selectively targeting an antigen on a surface of a cell with a compound, wherein the compound is according to any of the above embodiments.

[0049] In one embodiment, the cell is a mammalian cell. In one embodiment, the cell is a human cell.In one embodiment, the cell is a muscle cell.

[0050] In one embodiment, the antibody-drug conjugate is administered intravenously or subcutaneously.

[0051] In another aspect, the present disclosure provides a method of causing internalization of a compound by a myofiber, the method comprising contacting the myofiber with the antibody-drug conjugate of any of the above embodiments.

[0052] In another aspect, the present disclosure provides a method of producing a compound having a structure according to Formula (A):

$$Ab\text{-}(L1\text{-}B\text{-}L2\text{-}P)_n \quad (A),$$

or a pharmaceutically acceptable salt thereof, wherein:

Ab is an antigen-binding protein of any one of claims 1-14;

L1 is a first linker covalently bound to the side chain of a glutamine residue of the Ab;

B is a moiety comprising a triazole;

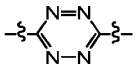
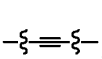
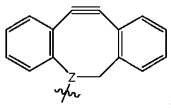
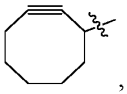
L2 is a second linker covalently bound to the therapeutic agent P;

P is a therapeutic agent selected from the group consisting of DHT and proDHT, and

n is an integer from 1 to 8, wherein the method comprises the steps of:

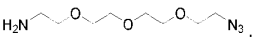
a) contacting, in the presence of a transglutaminase, the A comprising at least one glutamine residue with at least one compound L1-B';

b) contacting the product of step a) with one or more equivalents of a compound B"-L2-P, wherein the group B" is capable of covalently attaching to the group B',

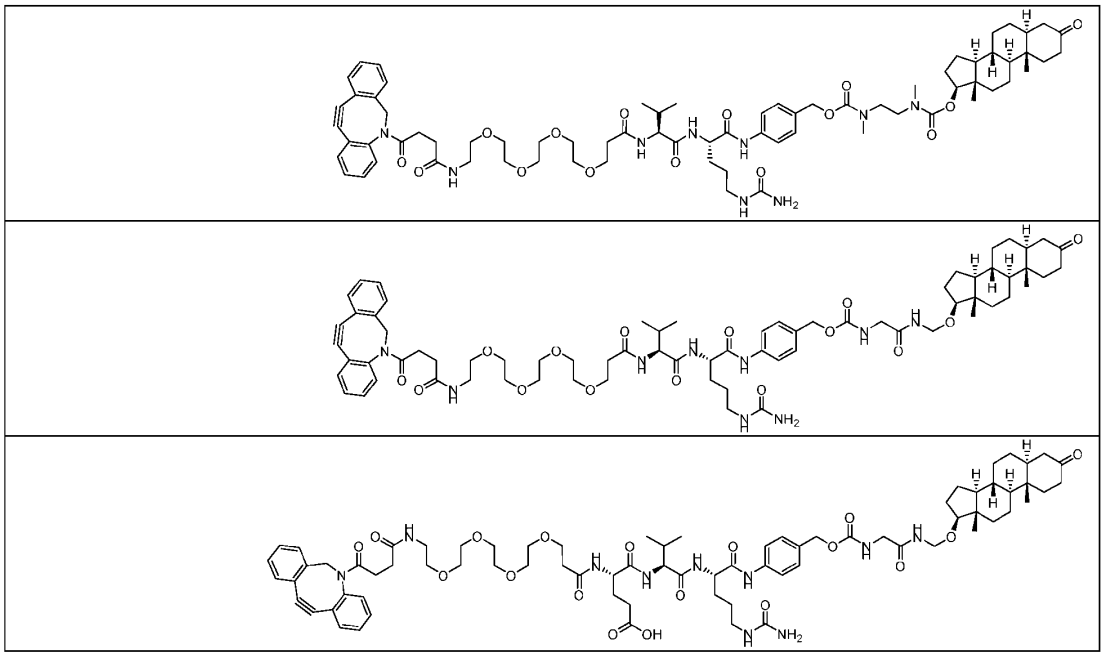
wherein one of the groups B' and B" is selected from $-N_3$ and  ; and the other of the groups B' and B" is selected from  ,  ; and  , where Z is C or N; and

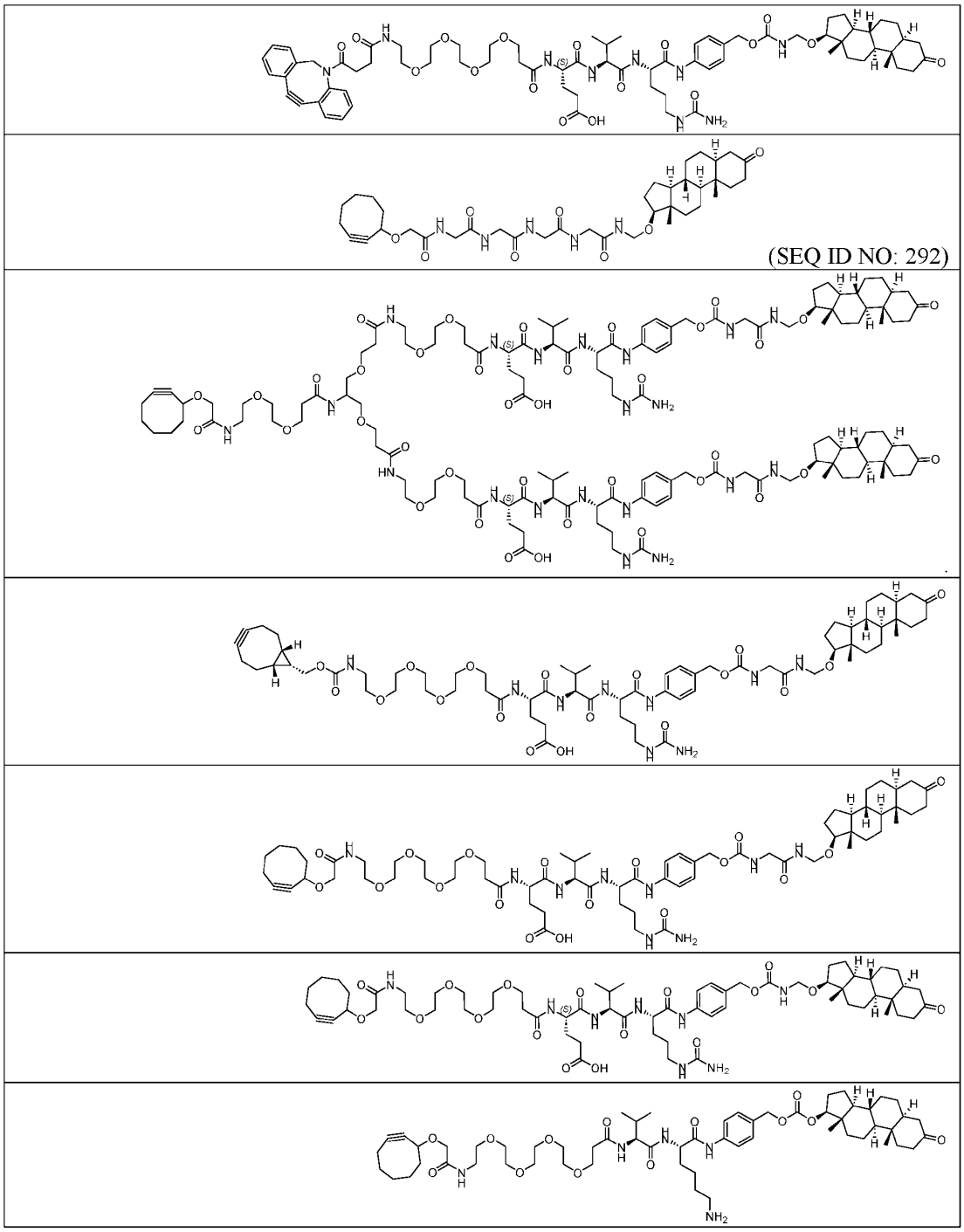
c) isolating the produced compound of Formula (A).

[0053] In one embodiment, the A has glutamine residues at positions 295 (Q295) and 297 (N297Q).

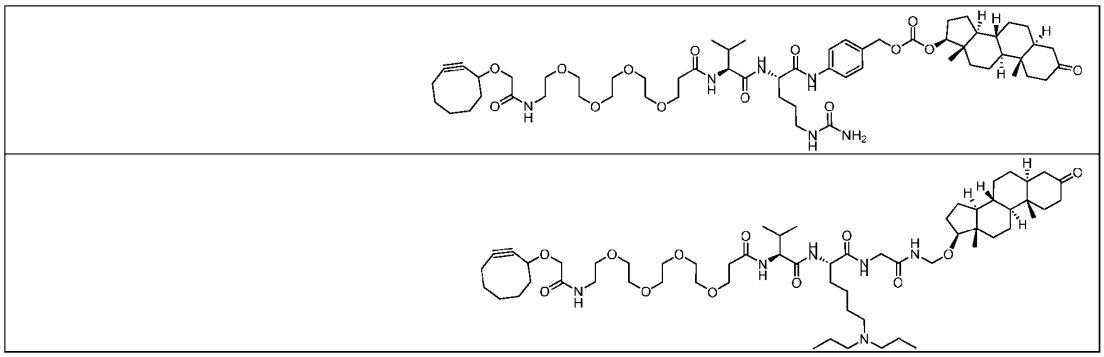
[0054] In one embodiment, the L1-B' has the structure  .

[0055] In one embodiment, the compound B"-L2-P has the structure selected from the group consisting of:

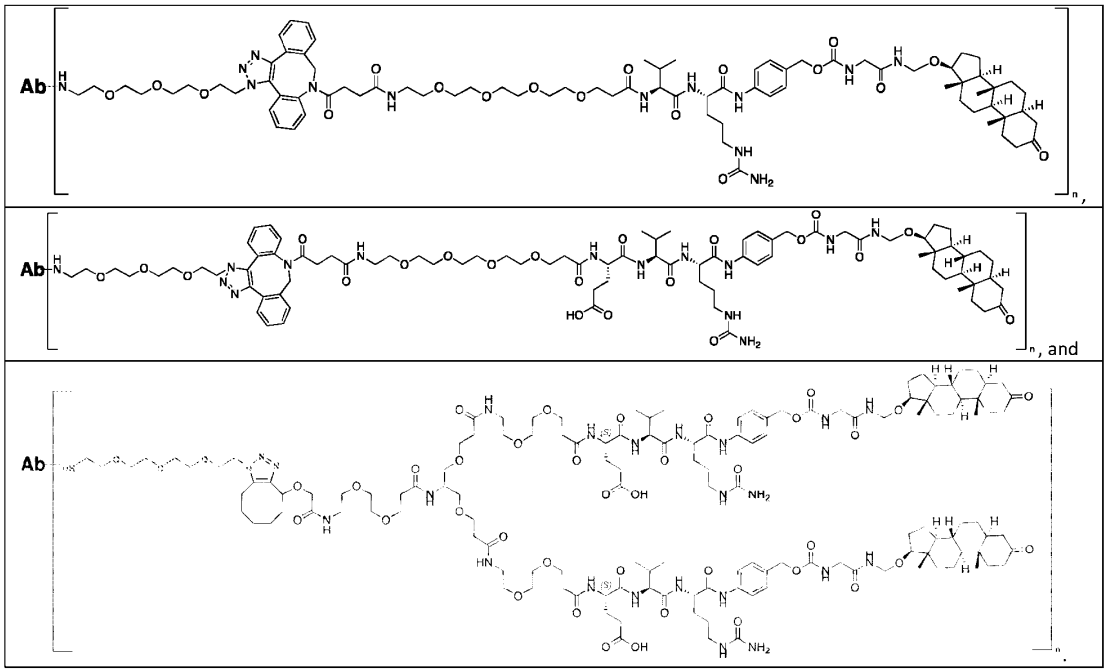




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[0056] In one embodiment, the compound of Formula (A) has the structure selected from the group consisting of:



[0057] In another aspect, the present disclosure provides a method of producing a compound having a structure according to Formula (I):

$$A - [L - P]_y \quad (I),$$

wherein A is the antigen-binding protein;

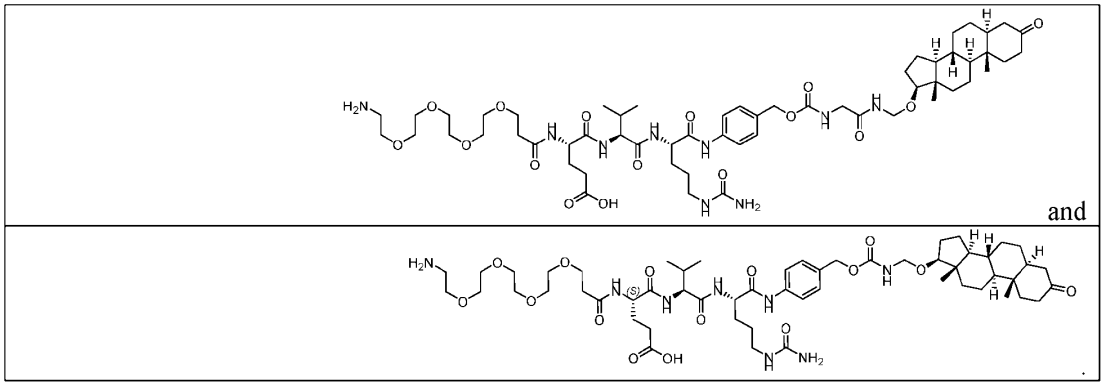
L is a linker;

P is a therapeutic agent selected from the group consisting of DHT and proDHT, and

y is an integer from 1 to 8, wherein the method comprises the steps of:

a) contacting, in the presence of a transglutaminase, the A comprising at least one glutamine residue with at least one compound L-P, wherein the compound L-P has at least one terminal amine moiety, and
b) isolating the produced compound of Formula (I).

[0058] In one embodiment, the compound L-P has the structure selected from the group consisting of:


and

[0059] These and other aspects of the present invention will become apparent to those skilled in the art after a reading of the following detailed description of the invention, including the appended claims.

DRAWINGS

[0060] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0061] **Figure 1** provides data regarding human myotube acetylcholine-induced calcium flux (relative light units; y-axis) after incubation with different concentrations (0.01 μM, 0.1 μM, 1 μM, and 10 μM; x-axis) of an anti-hCACNG1 antibody (anti-hCACNG1 Ab 1, anti-hCACNG1 Ab 2, or

anti-hCACNG1 Ab 3) or an isotype control antibody (Isotype control Ab 1, Isotype control Ab 2, or Isotype control Ab 3), or with 20 μM nicardipine as a positive control for calcium blocking. The anti-hCACNG1 antibodies tested here do not inhibit acetylcholine-induced calcium flux in human myotubes at these concentrations.

[0062] Figure 2 provides fluorescence immunohistochemistry images taken at 20x magnification of single myofibers *ex vivo* after isolation from wildtype ("WT") mice, mice that were homozygous for the deletion of CACNG1 ("KO"), or mice expressing only human CACNG1 ("*CACNG1*$^{Hu/Hu}$"); incubation with an anti-human CACNG1 antibody (anti-hCACNG1 Ab 4 or anti-hCACNG1 Ab 1), or an isotype control antibody (Isotype control Ab 5 or Isotype control Ab 2); and labelling with fluorescent-conjugated secondary antibodies. CACNG1 antibodies bound to CACNG1$^{Hu/Hu}$ myofibers, while isotype control antibodies did not.

[0063] Figure 3 provides single plane confocal fluorescence immunohistochemistry images taken at 20x magnification of single myofibers *ex vivo* after isolation from wildtype ("WT") mice, mice that were homozygous for the deletion of CACNG1 (KO), or mice expressing only human CACNG1 ("*CACNG1*$^{Hu/Hu}$"); and incubation with an anti-human CACNG1 antibody (anti-hCACNG1 Ab 2) or an isotype control antibody (Isotype control Ab 4) conjugated with Alexa 647 (A647) fluorophore for 30 minutes, 4 hours or 8 hours. Confocal imaging revealed that fluorophore-conjugated CACNG1 antibody bound to the surface of CACNG1$^{Hu/Hu}$ myofibers after 30 minutes of incubation, and that a portion of CACNG1 antibody was internalized and detected within the myofiber by 4 hours and 8 hours of incubation. Fluorophore-conjugated isotype control antibody was not detected to bind or internalize in CACNG1$^{Hu/Hu}$ myofibers.

[0064] Figure 4 provides cryo-fluorescence tomography images of a mouse 6 days after systemic injection of 10mg/kg of an anti-hCACNG1 antibody conjugated to Alexa 647 (anti-hCACNG1 Ab 2 or anti-hCACNG1 Ab 1) or an isotype control antibody conjugated to Alexa 647.

[0065] Figure 5 provides tiled fluorescence immunohistochemistry images taken at 20x magnification of images of gastrocnemius/plantaris/soleus, triceps, tibialis anterior, trapezius, diaphragm, pelvic floor muscles, or tongue sections of mice expressing only human CACNG1 ("*CACNG1*$^{Hu/Hu}$") after tail vein injection with 10mg/kg of an anti-human CACNG1 antibody (anti-hCACNG1 Ab 1 or anti-hCACNG1 Ab 2) or an isotype control antibody (Isotype control Ab 4) conjugated with Alexa 647 (A647) fluorophore and sacrifice 6 days post injection. Fluorophore-

conjugated CACNG1 antibody was detected in all of these skeletal muscles, with anti-hCACNG1 Ab 2 displaying a stronger signal in muscles compared to anti-hCACNG1 Ab 1. Only low levels of fluorescence were detected in muscles from isotype control and saline injected mice.

[0066] Figure 6 provides tiled fluorescence immunohistochemistry images taken at 20x magnification of images of liver, spleen, kidney, or brown adipose tissue sections of mice expressing only human CACNG1 ("*CACNG1*$^{Hu/Hu}$") after tail vein injection with 10mg/kg an anti-human CACNG1 antibody (anti-hCACNG1 Ab 1 or anti-hCACNG1 Ab 2) or an isotype control antibody (Isotype control Ab 4) conjugated with Alexa 647 (A647) fluorophore and sacrifice 6 days post injection. Neither fluorophore-conjugated CACNG1 antibody showed appreciable signal in these organs, with Alexa 647 levels similar to isotype and saline injected controls.

[0067] Figure 7 provides a schematic depicting an exemplary experimental timeline (top panel) and photomicrographs showing CACNG1 antibody distribution to the soleus muscle under sedentary and exercise conditions at either a 10mg/kg or a 50mg/kg (high) dose (bottom panel). CACNG1 distribution is altered by exercise and dose.

[0068] Figures 8A-8C depict schematics of various ADC conjugation routes. **Fig. 8A** provides a schematic of the two-step ADC conjugation procedure according to the present disclosure. **Fig. 8B** provides a schematic of the one-step ADC conjugation procedure according to the present disclosure. **Fig. 8C** provides a schematic of Cys-maleimide ADC conjugation.

[0069] Figures 9A-9B depict ES-MS (**Fig. 9A**) and the schematic structure (**Fig. 9B**) of aCACNG1-(**AL**)$_4$.

[0070] Figures 10A-10C depict ES-MS of aCACNG1-(**AL-LP2**)$_4$ ADC (**Fig. 10A**), ES-MS of FelD1-(**AL-LP2**)$_4$ ADC (**Fig. 10B**), and the schematic structure (**Fig. 10C**) of aCACNG1-(**AL-LP2**)$_4$.

[0071] Figures 11A-11C depict ES-MS spectra of aCACNG1-(**AL**)$_4$ (**Fig. 11A**), aCACNG1-(**AL-LP3**)$_4$ (**Fig. 11B**), and FelD1-(**AL-LP3**)$_4$ (**Fig. 11C**).

[0072] Figure 12 shows the results of the antagonist screen.

[0073] Figure 13 depicts in vitro plasma stability of anti-hCACNG1 Ab 5-L2 in plasma.

[0074] Figure 14 depicts in vitro plasma stability of anti-hCACNG1 Ab 5-L3 in plasma.

[0075] Figure 15 depicts the activation of the androgen receptor (24 hr) in LNCaP.hCANCG1.AR.Luc cell line after 24 hours of treatment.

[0076] Figure 16 depicts the activation of the androgen receptor (24 hr) in AR.Luc cell line after 24 hours of treatment. It shows the level of androgen receptor (AR) activation in terms of relative light units (RLU; y-axis) after a 24 hour incubation of an LNCaP cell line modified to express luciferase upon androgen receptor activation (AR.Luc) with: dihydrotestosterone (DHT) alone (unconjugated DHT); an anti-hCACNG1 antibody (anti-hCACNG1 Ab 6, anti-hCACNG1 Ab 7, anti-hCACNG1 Ab 8, anti-hCACNG1 Ab 9, anti-hCACNG1 Ab 10 or anti-hCACNG1 Ab 5) conjugated via a VC-PAB linker to DHT (L2); or an anti-FelD isotype control antibody (Isotype control Ab 1) conjugated via a VC-PAB linker to DHT (L2); at varying concentrations (Log[Conc. (M)]; x-axis). Only unconjugated DHT was shown to activate androgen receptor in this assay, while none of the CACNG1 antibodies conjugated to DHT showed any appreciable activation of the androgen receptor in this cell line that does not express hCACNG1.

[0077] Figure 17 depicts activation of the androgen receptor (48 and 72 hr) in hCACNG1.AR.Luc cell line after 24, 48, and 72 hours of treatment. It shows the level of androgen receptor (AR) activation in terms of relative light units (RLU; y-axis) after a 24 hour, 48 hour, or 72 hour incubation of a hCACNG1 expressing LNCaP cell line modified to also express luciferase upon androgen receptor activation (hCACNG1.AR.Luc) with: dihydrotestosterone (DHT) alone (unconjugated DHT); an anti-hCACNG1 antibody (anti-hCACNG1 Ab 6, anti-hCACNG1 Ab 7, anti-hCACNG1 Ab 8, anti-hCACNG1 Ab 9, anti-hCACNG1 Ab 10 or anti-hCACNG1 Ab 5) conjugated via a VC-PAB linker to DHT (L2); or an anti-FelD isotype control antibody (Isotype control Ab 1) conjugated via a VC-PAB linker to DHT (L2); at varying concentrations (Log[Conc. (M)]; x-axis). Several CACNG1 antibody-DHT conjugates activated androgen receptor in this hCACNG1 expressing cell line, and while the efficacy and potency of androgen receptor activation was lower than that of unconjugated at 24 hours following treatment, activation of the androgen receptor was sustained at 48 and 72 hours compared to unconjugated DHT.

DESCRIPTION

[0078] Provided herein are novel anti-human CACNG1 antibodies, and monovalent antigen binding fragments thereof, and antibody-drug conjugates (ADCs) which are useful in mediating

internalization of CACNG1. The anti-human CACNG1 antibodies, and monovalent antigen binding fragments thereof and ADCs comprising same may be useful, e.g., in the treatment of diseases, as part of multispecific antigen binding protein and/or multidomain therapeutic protein, and/or as an antibody drug conjugate.

[0079] The description herein is not limited to particular embodiments, compositions, methods and experimental conditions described, as such embodiments, compositions, methods and conditions may vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0080] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing as described herein, some preferred methods and materials are now described. All publications cited herein are incorporated herein by reference to describe in their entirety. 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.

[0081] The term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. For example, the expression "about 100" includes 99 and 101 and all values in between (e.g., 99.1, 99.2, 99.3, 99.4, etc.).

[0082] Voltage-dependent calcium channels are generally composed of five subunits. The protein encoded by the *CACNG1* gene represents one of these subunits. "CACNG1" includes a protein encoded by the *CACNG1* gene, and is one of two known gamma subunit proteins. CACNG1 is part of the skeletal muscle 1,4-dihydropyridine-sensitive calcium channel and is an integral membrane protein that plays a role in excitation-contraction coupling. CACNG1 is part of a functionally diverse eight-member protein subfamily of the PMP-22/EMP/MP20 family and is located in a cluster with two family members that function as transmembrane AMPA receptor regulatory proteins (TARPs). CACNG1 is highly and specifically expressed in skeletal muscle. The gene encoding human CACNG1 (*CACNG1*) is located on the long arm of chromosome 17. *CACNG1* comprises 4 exons and is approximately 12,244 bases long. An exemplary sequence for human *CACNG1* gene is assigned NCBI Accession Number NM_000727.4 (SEQ ID NO: 241). An exemplary human CACNG1 protein is assigned NCBI Accession Number NP_000718 (SEQ ID NO: 241).

[0083] The phrase "an antibody that binds CACNG1" or an "anti-hCACNG1 antibody" includes an antibody and antigen-binding fragment thereof that specifically recognizes a single CACNG1 molecule. An antibody and antigen-binding fragment thereof as described herein may bind soluble CACNG1 and/or cell surface expressed CACNG1. Soluble CACNG1 includes natural CACNG1 proteins as well as recombinant CACNG1 protein variants that lack a transmembrane domain or are otherwise unassociated with a cell membrane.

[0084] The expression "cell surface-expressed CACNG1" refers to one or more CACNG1 protein(s) that is/are expressed on the surface of a cell *in vitro* or *in vivo*, such that at least a portion of a CACNG1 protein is exposed to the extracellular side of the cell membrane and is accessible to an antigen-binding portion of an antibody. A "cell surface-expressed CACNG1" can comprise or consist of a CACNG1 protein expressed on the surface of a cell which normally expresses CACNG1 protein. Alternatively, "cell surface-expressed CACNG1" can comprise or consist of a CACNG1 protein expressed on the surface of a cell that normally does not express human CACNG1 on its surface but has been artificially engineered to express CACNG1 on its surface.

[0085] The term "antigen-binding molecule" includes an antibody and an antigen-binding fragment of an antibody.

[0086] The term "antibody" refers to any antigen-binding molecule or molecular complex comprising at least one complementarity determining region (CDR) that specifically binds to or interacts with a particular antigen (*e.g.*, CACNG1). The term "antibody", as used herein, includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as multimers thereof (e.g., IgM). Each heavy chain comprises a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region comprises 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 (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4 (heavy chain CDRs may be abbreviated as HCDR1, HCDR2 and

HCDR3; light chain CDRs may be abbreviated as LCDR1, LCDR2 and LCDR3. The term "high affinity" antibody refers to those antibodies having a binding affinity to their target of at least 10^{-9} M, at least 10^{-10} M; at least 10^{-11} M; or at least 10^{-12} M, as measured by surface plasmon resonance, *e.g.*, BIACORE™ or solution-affinity ELISA. The term "antibody" may encompass any type of antibody, such as e.g. monoclonal or polyclonal. Moreover, the antibody may be or any origin, such as e.g. mammalian or non-mammalian. In one embodiment, the antibody may be mammalian or avian. In a further embodiment, the antibody may be of human origin and may further be a human monoclonal antibody.

[0087] The term "antibody" also includes antigen-binding fragments of full antibody molecules. The terms "antigen-binding portion" of an antibody, "antigen-binding fragment" of an antibody, and the like include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen to form a complex. Antigen-binding fragments of an antibody may be derived, *e.g.*, from full antibody molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding antibody variable and optionally constant domains. Such DNA is known and/or is readily available from, *e.g.*, commercial sources, DNA libraries (including, *e.g.*, phage-antibody libraries), or can be synthesized. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add or delete amino acids, etc.

[0088] Non-limiting examples of antigen-binding fragments include: (i) Fab fragments; (ii) F(ab')2 fragments; (iii) Fd fragments; (iv) Fv fragments; (v) single-chain Fv (scFv) molecules; (vi) dAb fragments; and (vii) minimal recognition units consisting of the amino acid residues that mimic the hypervariable region of an antibody (*e.g.*, an isolated complementarity determining region (CDR) such as a CDR3 peptide), or a constrained FR3-CDR3-FR4 peptide. Other engineered molecules, such as domain-specific antibodies, single domain antibodies, domain-deleted antibodies, chimeric antibodies, CDR-grafted antibodies, diabodies, triabodies, tetrabodies, minibodies, nanobodies (*e.g.* monovalent nanobodies, bivalent nanobodies, etc.), small modular immunopharmaceuticals (SMIPs), and shark variable IgNAR domains, are also encompassed within the expression "antigen-binding fragment".

[0089] An antigen-binding fragment of an antibody will typically comprise at least one variable domain. The variable domain may be of any size or amino acid composition and will generally comprise at least one CDR which is adjacent to or in frame with one or more framework sequences. In antigen-binding fragments having a V_H domain associated with a V_L domain, the V_H and V_L domains may be situated relative to one another in any suitable arrangement. For example, the variable region may be dimeric and contain V_H-V_H, V_H-V_L or V_L-V_L dimers. Alternatively, the antigen-binding fragment of an antibody may contain a monomeric V_H or V_L domain.

[0090] In certain embodiments, an antigen-binding fragment of an antibody may contain at least one variable domain covalently linked to at least one constant domain. Non-limiting, exemplary configurations of variable and constant domains that may be found within an antigen-binding fragment of an antibody as described herein include: (i) V_H-C_H1; (ii) V_H-C_H2; (iii) V_H-C_H3; (iv) V_H-C_H1-C_H2; (v) V_H-C_H1-C_H2-C_H3; (vi) V_H-C_H2-C_H3; (vii) V_H-C_L; (viii) V_L-C_H1; (ix) V_L-C_H2; (x) V_L-C_H3; (xi) V_L-C_H1-C_H2; (xii) V_L-C_H1-C_H2-C_H3; (xiii) V_L-C_H2-C_H3; and (xiv) V_L-C_L. In any configuration of variable and constant domains, including any of the exemplary configurations listed above, the variable and constant domains may be either directly linked to one another or may be linked by a full or partial hinge or linker region. A hinge region may consist of at least 2 (*e.g.*, 5, 10, 15, 20, 40, 60 or more) amino acids which result in a flexible or semi-flexible linkage between adjacent variable and/or constant domains in a single polypeptide molecule. Moreover, an antigen-binding fragment of an antibody as described herein may comprise a homo-dimer or hetero-dimer (or other multimer) of any of the variable and constant domain configurations listed above in non-covalent association with one another and/or with one or more monomeric V_H or V_L domain (*e.g.*, by disulfide bond(s)).

[0091] As with full antibody molecules, antigen-binding fragments may be monospecific or multispecific (*e.g.*, bispecific). A multispecific antigen-binding fragment of an antibody will typically comprise at least two different variable domains, wherein each variable domain is capable of specifically binding to a separate antigen or to a different epitope on the same antigen. Any multispecific antibody format, including the exemplary bispecific antibody formats disclosed herein, may be adapted for use in the context of an antigen-binding fragment of an antibody as described herein using routine techniques available in the art.

[0092] In certain embodiments, the anti-hCACNG1 antibodies as described herein are human antibodies. The term "human antibody" refers to antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies as described herein may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for example in the CDRs and in particular CDR3. However, the term "human antibody" is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

[0093] The antibodies as described herein may, in some embodiments, be recombinant human antibodies. The term "recombinant human antibody" is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes (see e.g., Taylor et al. (1992) Nucl. Acids Res. 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

[0094] Human antibodies may exist in two general forms that are associated with hinge heterogeneity. In one general form, an immunoglobulin molecule comprises a stable four chain construct of approximately 150-160 kDa in which the dimers are held together by an interchain heavy chain disulfide bond. In a second general form, the dimers are not linked via inter-chain disulfide bonds and a molecule of about 75-80 kDa is formed composed of a covalently coupled light and heavy chain (half-antibody). These forms have been extremely difficult to separate, even after affinity purification.

[0095] The frequency of appearance of the second form in various intact IgG isotypes is due to, but not limited to, structural differences associated with the hinge region isotype of the antibody. A single amino acid substitution in the hinge region of the human IgG4 hinge can significantly reduce the appearance of the second form (Angal et al. (1993) Molecular Immunology 30:105) to levels typically observed using a human IgG1 hinge. The antibodies as described herein may have one or more mutations in the hinge, C_H2 or C_H3 region which may be desirable, for example, in production, to improve the yield of the desired antibody form.

[0096] The antibodies as described herein may be isolated antibodies. An "isolated antibody" refers to an antibody that has been identified and separated and/or recovered from at least one component of its natural environment. For example, an antibody that has been separated or removed from at least one component of an organism, or from a tissue or cell in which the antibody naturally exists or is naturally produced, may be considered an "isolated antibody." An isolated antibody also includes an antibody *in situ* within a recombinant cell. Isolated antibodies are antibodies that have been subjected to at least one purification or isolation step. According to certain embodiments, an isolated antibody may be substantially free of other cellular material and/or chemicals.

[0097] Also described herein are one-arm antibodies that bind CACNG1. The term "one-arm antibody" refers to an antigen-binding molecule comprising a single antibody heavy chain and a single antibody light chain. The one-arm antibodies as described herein may comprise any of the HCVR/LCVR or CDR amino acid sequences as set forth in Table 1.

[0098] The anti-hCACNG1 antibodies disclosed herein may comprise one or more amino acid substitutions, insertions and/or deletions in the framework and/or CDR regions of the heavy and light chain variable domains as compared to the corresponding germline sequences from which the antibodies were derived. Such mutations can be readily ascertained by comparing the amino acid sequences disclosed herein to germline sequences available from, for example, public antibody sequence databases. Also described herein are antibodies, and antigen-binding fragments thereof, which are derived from any of the amino acid sequences disclosed herein, wherein one or more amino acids within one or more framework and/or CDR regions are mutated to the corresponding residue(s) of the germline sequence from which the antibody was derived, or to the corresponding residue(s) of another human germline sequence, or to a conservative amino acid substitution of the

corresponding germline residue(s) (such sequence changes are referred to herein collectively as "germline mutations"). A person of ordinary skill in the art, starting with the heavy and light chain variable region sequences disclosed herein, can easily produce numerous antibodies and antigen-binding fragments which comprise one or more individual germline mutations or combinations thereof. In certain embodiments, all of the framework and/or CDR residues within the V_H and/or V_L domains are mutated back to the residues found in the original germline sequence from which the antibody was derived. In other embodiments, only certain residues are mutated back to the original germline sequence, *e.g.*, only the mutated residues found within the first 8 amino acids of FR1 or within the last 8 amino acids of FR4, or only the mutated residues found within CDR1, CDR2 or CDR3. In other embodiments, one or more of the framework and/or CDR residue(s) are mutated to the corresponding residue(s) of a different germline sequence (*i.e.*, a germline sequence that is different from the germline sequence from which the antibody was originally derived). Furthermore, the antibodies as described herein may contain any combination of two or more germline mutations within the framework and/or CDR regions, *e.g.*, wherein certain individual residues are mutated to the corresponding residue of a particular germline sequence while certain other residues that differ from the original germline sequence are maintained or are mutated to the corresponding residue of a different germline sequence. Once obtained, an antibody and an antigen-binding fragment that contains one or more germline mutations can be easily tested for one or more desired property such as, improved binding specificity, increased binding affinity, improved or enhanced antagonistic or agonistic biological properties (as the case may be), reduced immunogenicity, etc. In some embodiments, an antibody or an antigen-binding fragment as described herein is obtained in this general manner.

[0099] Also described herein are anti-hCACNG1 antibodies comprising variants of any of the HCVR, LCVR, and/or CDR amino acid sequences disclosed herein having one or more conservative substitutions. For example, some embodiments include anti-hCACNG1 antibodies having HCVR, LCVR, and/or CDR amino acid sequences with, *e.g.*, 10 or fewer, 8 or fewer, 6 or fewer, 4 or fewer, etc. conservative amino acid substitutions relative to any of the HCVR, LCVR, and/or CDR amino acid sequences set forth in Table 1 herein.

[0100] The phrase "bispecific antibody" includes an antibody capable of selectively binding two or more epitopes. Bispecific antibodies generally comprise two different heavy chains, with each heavy chain specifically binding a different epitope—either on two different molecules (*e.g.*,

antigens) or on the same molecule (*e.g.*, on the same antigen). If a bispecific antibody is capable of selectively binding two different epitopes (a first epitope and a second epitope), the affinity of the first heavy chain for the first epitope will generally be at least one to two or three or four orders of magnitude lower than the affinity of the first heavy chain for the second epitope, and vice versa. The epitopes recognized by the bispecific antibody can be on the same or a different target (*e.g.*, on the same or a different protein). Bispecific antibodies can be made, for example, by combining heavy chains that recognize different epitopes of the same antigen. For example, nucleic acid sequences encoding heavy chain variable sequences that recognize different epitopes of the same antigen can be fused to nucleic acid sequences encoding different heavy chain constant regions, and such sequences can be expressed in a cell that expresses an immunoglobulin light chain. A typical bispecific antibody has two heavy chains each having three heavy chain CDRs, followed by (N-terminal to C-terminal) a CH1 domain, a hinge, a CH2 domain, and a CH3 domain, and an immunoglobulin light chain that either does not confer antigen-binding specificity but that can associate with each heavy chain, or that can associate with each heavy chain and that can bind one or more of the epitopes bound by the heavy chain antigen-binding regions, or that can associate with each heavy chain and enable binding or one or both of the heavy chains to one or both epitopes.

[0101] The phrase "heavy chain," or "immunoglobulin heavy chain" includes an immunoglobulin heavy chain constant region sequence from any organism, and unless otherwise specified includes a heavy chain variable domain. Heavy chain variable domains include three heavy chain CDRs and four FR regions, unless otherwise specified. Fragments of heavy chains include CDRs, CDRs and FRs, and combinations thereof. A typical heavy chain has, following the variable domain (from N-terminal to C-terminal), a CH1 domain, a hinge, a CH2 domain, and a CH3 domain. A functional fragment of a heavy chain includes a fragment that is capable of specifically recognizing an antigen (*e.g.*, recognizing the antigen with a KD in the micromolar, nanomolar, or picomolar range), that is capable of expressing and secreting from a cell, and that comprises at least one CDR.

[0102] The phrase "light chain" includes an immunoglobulin light chain constant region sequence from any organism, and unless otherwise specified includes human kappa and lambda light chains. Light chain variable (VL) domains typically include three light chain CDRs and four framework (FR) regions, unless otherwise specified. Generally, a full-length light chain includes, from amino terminus to carboxyl terminus, a VL domain that includes FR1-CDR1- FR2-CDR2-FR3-CDR3-

FR4, and a light chain constant domain. Light chains that may be useful include *e.g.*, those, that do not selectively bind either the first or second antigen selectively bound by the antigen-binding protein. Suitable light chains include those that can be identified by screening for the most commonly employed light chains in existing antibody libraries (wet libraries or *in silico*), where the light chains do not substantially interfere with the affinity and/or selectivity of the antigen-binding domains of the antigen-binding proteins. Suitable light chains include those that can bind one or both epitopes that are bound by the antigen-binding regions of the antigen-binding protein.

[0103] The phrase "variable domain" includes an amino acid sequence of an immunoglobulin light or heavy chain (modified as desired) that comprises the following amino acid regions, in sequence from N-terminal to C-terminal (unless otherwise indicated): FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. A "variable domain" includes an amino acid sequence capable of folding into a canonical domain (VH or VL) having a dual beta sheet structure wherein the beta sheets are connected by a disulfide bond between a residue of a first beta sheet and a second beta sheet.

[0104] The phrase "complementarity determining region," or the term "CDR," includes an amino acid sequence encoded by a nucleic acid sequence of an organism's immunoglobulin genes that normally (*i.e.*, in a wildtype animal) appears between two framework regions in a variable region of a light or a heavy chain of an immunoglobulin molecule (*e.g.*, an antibody or a T cell receptor). A CDR can be encoded by, for example, a germline sequence or a rearranged or unrearranged sequence, and, for example, by a naive or a mature B cell or a T cell. In some circumstances (*e.g.*, for a CDR3), CDRs can be encoded by two or more sequences (*e.g.*, germline sequences) that are not contiguous (*e.g.*, in an unrearranged nucleic acid sequence) but are contiguous in a B cell nucleic acid sequence, *e.g.*, as the result of splicing or connecting the sequences (*e.g.*, V-D-J recombination to form a heavy chain CDR3).

[0105] The term "antibody fragment", refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. Examples of binding fragments encompassed within the term "antibody fragment" include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.* (1989) Nature 241:544-546), which consists of a VH

domain, (vi) an isolated CDR, and (vii) an scFv, which consists of the two domains of the Fv fragment, VL and VH, joined by a synthetic linker to form a single protein chain in which the VL and VH regions pair to form monovalent molecules. Other forms of single chain antibodies, such as diabodies are also encompassed under the term "antibody" (see *e.g.*, Holliger *et al.* (1993) PNAS USA 90:6444-6448; Poljak *et al.* (1994) Structure 2:1121-1123).

[0106] The phrase "Fc-containing protein" includes antibodies, bispecific antibodies, immunoadhesins, and other binding proteins that comprise at least a functional portion of an immunoglobulin CH2 and CH3 region. A "functional portion" refers to a CH2 and CH3 region that can bind a Fc receptor (*e.g.*, an FcyR; or an FcRn, *i.e.*, a neonatal Fc receptor), and/or that can participate in the activation of complement. If the CH2 and CH3 region contains deletions, substitutions, and/or insertions or other modifications that render it unable to bind any Fc receptor and also unable to activate complement, the CH2 and CH3 region is not functional.

[0107] Fc-containing proteins can comprise modifications in immunoglobulin domains, including where the modifications affect one or more effector function of the binding protein (*e.g.*, modifications that affect FcyR binding, FcRn binding and thus half-life, and/or CDC activity). Such modifications include, but are not limited to, the following modifications and combinations thereof, with reference to EU numbering of an immunoglobulin constant region: 238, 239, 248, 249, 250, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 297, 298, 301, 303, 305, 307, 308, 309, 311, 312, 315, 318, 320, 322, 324, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 337, 338, 339, 340, 342, 344, 356, 358, 359, 360, 361, 362, 373, 375, 376, 378, 380, 382, 383, 384, 386, 388, 389, 398, 414, 416, 419, 428, 430, 433, 434, 435, 437, 438, and 439.

[0108] For example, and not by way of limitation, the binding protein is an Fc-containing protein and exhibits enhanced serum half-life (as compared with the same Fc-containing protein without the recited modification(s)) and have a modification at position 250 (*e.g.*, E or Q); 250 and 428 (*e.g.*, L or F); 252 (*e.g.*, L/Y/F/W or T), 254 (*e.g.*, S or T), and 256 (*e.g.*, S/R/Q/E/D or T); or a modification at 428 and/or 433 (*e.g.*, L/R/SI/P/Q or K) and/or 434 (*e.g.*, H/F or Y); or a modification at 250 and/or 428; or a modification at 307 or 308 (*e.g.*, 308F, V308F), and 434. In another example, the modification can comprise a 428L (*e.g.*, M428L) and 434S (*e.g.*, N434S) modification; a 428L, 2591 (*e.g.*, V259I), and a 308F (*e.g.*, V308F) modification; a 433K (*e.g.*,

H433K) and a 434 (*e.g.*, 434Y) modification; a 252, 254, and 256 (*e.g.*, 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.*, T250Q and M428L); a 307 and/or 308 modification (*e.g.*, 308F or 308P).

[0109] The term "antigen-binding protein," as used herein, refers to a polypeptide or protein (one or more polypeptides complexed in a functional unit) that specifically recognizes an epitope on an antigen, such as a cell-specific antigen and/or a target antigen as described herein. An antigen-binding protein may be multi-specific. The term "multi-specific" with reference to an antigen-binding protein means that the protein recognizes different epitopes, either on the same antigen or on different antigens. A multi-specific antigen-binding protein as described herein can be a single multifunctional polypeptide, or it can be a multimeric complex of two or more polypeptides that are covalently or non-covalently associated with one another. The term "antigen-binding protein" includes antibodies or fragments thereof as described herein that may be linked to or co-expressed with another functional molecule, *e.g.*, another peptide or protein. For example, an antibody or fragment thereof can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, non-covalent association or otherwise) to one or more other molecular entities, such as a protein or fragment thereof to produce a bispecific or a multi-specific antigen-binding molecule with a second binding specificity.

[0110] The term "protein" means any amino acid polymer having more than about 20 amino acids covalently linked via amide bonds. Proteins contain one or more amino acid polymer chains, generally known in the art as "polypeptides". Thus, a polypeptide may be a protein, and a protein may contain multiple polypeptides to form a single functioning biomolecule. Disulfide bridges (*i.e.*, between cysteine residues to form cystine) may be present in some proteins. These covalent links may be within a single polypeptide chain, or between two individual polypeptide chains. For example, disulfide bridges are essential to proper structure and function of insulin, immunoglobulins, protamine, and the like. For a recent review of disulfide bond formation, see Oka and Bulleid, "Forming disulfides in the endoplasmic reticulum," 1833(11) Biochim Biophys Acta 2425-9 (2013).

[0111] As used herein, "protein" includes biotherapeutic proteins, recombinant proteins used in research or therapy, trap proteins and other Fc-fusion proteins, chimeric proteins, antibodies, monoclonal antibodies, human antibodies, bispecific antibodies, antibody fragments, nanobodies,

recombinant antibody chimeras, scFv fusion proteins, cytokines, chemokines, peptide hormones, and the like. Proteins may be produced using recombinant cell-based production systems, such as the insect bacculovirus system, yeast systems (*e.g.*, *Pichia* sp.), mammalian systems (*e.g.*, CHO cells and CHO derivatives like CHO-K1 cells). For a recent review discussing biotherapeutic proteins and their production, see Ghaderi *et al.*, "Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation," 28 Biotechnol Genet Eng Rev. 147-75 (2012).

[0112] As used herein, the term "epitope" refers to the portion of the antigen which is recognized by the multi-specific antigen-binding polypeptide. A single antigen (such as an antigenic polypeptide) may have more than one epitope. Epitopes may be defined as structural or functional. Functional epitopes are generally a subset of structural epitopes and are defined as those residues that directly contribute to the affinity of the interaction between the antigen-binding polypeptide and the antigen. Epitopes may also be conformational, that is, composed of non-linear amino acids. In certain embodiments, epitopes may include determinants that are chemically active surface groupings of molecules such as amino acids, sugar side chains, phosphoryl groups, or sulfonyl groups, and, in certain embodiments, may have specific three-dimensional structural characteristics, and/or specific charge characteristics. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents.

[0113] The term "domain" refers to any part of a protein or polypeptide having a particular function or structure. Preferably, domains as described herein bind to cell-specific or target antigens. Cell-specific antigen- or target antigen-binding domains, and the like, as used herein, include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen.

[0114] The term "half-body" or "half-antibody", which are used interchangeably, refers to half of an antibody, which essentially contains one heavy chain and one light chain. Antibody heavy chains can form dimers, thus the heavy chain of one half-body can associate with heavy chain associated with a different molecule (*e.g.*, another half-body) or another Fc-containing polypeptide. Two slightly different Fc-domains may "heterodimerize" as in the formation of bispecific antibodies or other heterodimers, -trimers, -tetramers, and the like. See Vincent and Murini,

"Current strategies in antibody engineering: Fc engineering and pH-dependent antigen binding, bispecific antibodies and antibody drug conjugates," 7 Biotechnol. J. 1444-1450 (20912); and Shimamoto *et al.*, "Peptibodies: A flexible alternative format to antibodies," 4(5) MAbs 586-91 (2012).

[0115] The term "single-chain variable fragment" or "scFv" includes a single chain fusion polypeptide containing an immunoglobulin heavy chain variable region (VH) and an immunoglobulin light chain variable region (VL). In some embodiments, the VH and VL are connected by a linker sequence of 10 to 25 amino acids. ScFv polypeptides may also include other amino acid sequences, such as CL or CH1 regions. ScFv molecules can be manufactured by phage display or made by directly subcloning the heavy and light chains from a hybridoma or B-cell. Ahmad *et al.,* Clinical and Developmental Immunology, volume 2012, article ID 98025 is incorporated herein by reference for methods of making scFv fragments by phage display and antibody domain cloning.

[0116] The terms "treat" or "treatment" of a state, disorder or condition include: (1) preventing, delaying, or reducing the incidence and/or likelihood of the appearance of at least one clinical or sub-clinical symptom of the state, disorder or condition developing in a subject that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; or (2) inhibiting the state, disorder or condition, i.e., arresting, reducing or delaying the development of the disease or a relapse thereof or at least one clinical or sub-clinical symptom thereof; or (3) relieving the disease, i.e., causing regression of the state, disorder or condition or at least one of its clinical or sub-clinical symptoms. The benefit to a subject to be treated is either statistically significant or at least perceptible to the patient or to the physician. In some embodiments, treatment comprises methods wherein cells are ablated in such manner where disease is indirectly impacted. In certain embodiments, treatment comprises depleting immune cells as a hematopoietic conditioning regimen prior to therapy.

[0117] A "subject" or "patient" or "individual" or "animal", as used herein, refers to humans, veterinary animals (e.g., cats, dogs, cows, horses, sheep, pigs, etc.) and experimental animal models of diseases (e.g., mice, rats). In a preferred embodiment, the subject is a human.

[0118] As used herein the term "effective" applied to dose or amount refers to that quantity of a compound or pharmaceutical composition that is sufficient to result in a desired activity upon administration to a subject in need thereof. Note that when a combination of active ingredients is administered, the effective amount of the combination may or may not include amounts of each ingredient that would have been effective if administered individually. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, the particular drug or drugs employed, the mode of administration, and the like.

[0119] The phrase "pharmaceutically acceptable salt", as used in connection with compositions of the disclosure, refers to any salt suitable for administration to a patient. Suitable salts include, but are not limited to, those disclosed in Berge et al., "Pharmaceutical Salts", *J. Pharm. Sci.*, 1977, 66:1, incorporated herein by reference. Examples of salts include, but are not limited to, acid derived, base derived, organic, inorganic, amine, and alkali or alkaline earth metal salts, including but not limited to calcium salts, magnesium salts, potassium salts, sodium salts, salts of hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid, acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methane sulfonic acid, ethane sulfonic acid, p toluene sulfonic acid, salicylic acid, and the like. In some examples, a payload described herein (e.g., a rifamycin analog described herein) comprises a tertiary amine, where the nitrogen atom in the tertiary amine is the atom through which the payload is bonded to a linker or a linker-spacer. In such instances, bonding to the tertiary amine of the payload yields a quaternary amine in the linker-payload molecule. The positive charge on the quaternary amine can be balanced by a counter ion (e.g., chloro, bromo, iodo, or any other suitably charged moiety such as those described herein).

[0120] By "comprising" or "containing" or "including" is meant that at least the named compound, element, particle, or method step is present in the composition or article or method, but does not exclude the presence of other compounds, materials, particles, or method steps, even if the other such compounds, material, particles, or method steps have the same function as what is named.

[0121] Compounds of the present disclosure include those described generally herein, and are further illustrated by the classes, subclasses, and species disclosed herein. As used herein, the

following definitions shall apply unless otherwise indicated. For purposes of this disclosure, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics, 75th Ed. Additionally, general principles of organic chemistry are described in "Organic Chemistry", Thomas Sorrell, University Science Books, Sausalito: 1999, and "March's Advanced Organic Chemistry", 5th Ed., Ed.: Smith, M.B. and March, J., John Wiley & Sons, New York: 2001, the entire contents of which are hereby incorporated by reference.

[0122] As used herein, the term "alkyl" is given its ordinary meaning in the art and may include saturated aliphatic groups, including straight-chain alkyl groups, branched-chain alkyl groups, cycloalkyl (alicyclic) groups, alkyl substituted cycloalkyl groups, and cycloalkyl substituted alkyl groups. In certain embodiments, a straight chain or branched chain alkyl has about 1–20 carbon atoms in its backbone (e.g., C_1–C_{20} for straight chain, C_2–C_{20} for branched chain), and alternatively, about 1–10 carbon atoms, or about 1 to 6 carbon atoms. In some embodiments, a cycloalkyl ring has from about 3–10 carbon atoms in their ring structure where such rings are monocyclic or bicyclic, and alternatively about 5, 6 or 7 carbons in the ring structure. In some embodiments, an alkyl group may be a lower alkyl group, wherein a lower alkyl group comprises 1–4 carbon atoms (e.g., C_1–C_4 for straight chain lower alkyls).

[0123] As used herein, the term "alkenyl" refers to an alkyl group, as defined herein, having one or more double bonds.

[0124] As used herein, the term "alkynyl" refers to an alkyl group, as defined herein, having one or more triple bonds.

[0125] The term "heteroatom" means one or more of oxygen, sulfur, nitrogen, phosphorus, or silicon (including, any oxidized form of nitrogen, sulfur, phosphorus, or silicon; the quaternized form of any basic nitrogen or; a substitutable nitrogen of a heterocyclic ring.

[0126] The term "halogen" means F, Cl, Br, or I; the term "halide" refers to a halogen radical or substituent, namely -F, -Cl, -Br, or -I.

[0127] The term "adduct", e.g., "an adduct of group B'" of the present disclosure encompasses any moiety comprising the product of an addition reaction, e.g., an addition reaction of group B', independent of the synthetic steps taken to produce the moiety.

[0128] The term "covalent attachment" means formation of a covalent bond, i.e., a chemical bond that involves sharing of one or more electron pairs between two atoms. Covalent bonding may include different interactions, including but not limited to σ-bonding, π-bonding, metal-to-metal bonding, agostic interactions, bent bonds, and three-center two-electron bonds. When a first group is said to be "capable of covalently attaching" to a second group, this means that the first group is capable of forming a covalent bond with the second group, directly or indirectly, e.g., through the use of a catalyst or under specific reaction conditions. Non-limiting examples of groups capable of covalently attaching to each other may include, e.g., an amine and a carboxylic acid (forming an amide bond), a diene and a dienophile (via a Diels-Alder reaction), and an azide and an alkyne (forming a triazole via a 1,3-cycloaddition reaction).

[0129] As described herein, compounds of the disclosure may contain "optionally substituted" moieties. In general, the term "substituted," whether preceded by the term "optionally" or not, means that one or more hydrogens of the designated moiety are replaced with a suitable substituent. Unless otherwise indicated, an "optionally substituted" group may have a suitable substituent at each substitutable position of the group, and when more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. Combinations of substituents envisioned by this disclosure are preferably those that result in the formation of stable or chemically feasible compounds. The term "stable," as used herein, refers to compounds that are not substantially altered when subjected to conditions to allow for their production, detection, and, in certain embodiments, their recovery, purification, and use for one or more of the purposes disclosed herein.

[0130] Unless otherwise stated, structures depicted herein are also meant to include all isomeric (e.g., enantiomeric, diastereomeric, and geometric (or conformational)) forms of the structure; for example, the R and S configurations for each asymmetric center, (Z) and (E) double bond isomers, and (Z) and (E) conformational isomers. Therefore, single stereochemical isomers as well as enantiomeric, diastereomeric, and geometric (or conformational) mixtures of the present compounds are within the scope of the disclosure.

[0131] Unless otherwise stated, all tautomeric forms of the compounds of the disclosure are within the scope of the disclosure.

[0132] Additionally, unless otherwise stated, structures depicted herein are also meant to include compounds that differ only in the presence of one or more isotopically enriched atoms. For example, compounds having the present structures except for the replacement of hydrogen by deuterium or tritium, or the replacement of a carbon by a ^{11}C- or ^{13}C- or ^{14}C -enriched carbon are within the scope of this disclosure.

[0133] It is also to be understood that the mention of one or more method steps does not preclude the presence of additional method steps or intervening method steps between those steps expressly identified. Similarly, it is also to be understood that the mention of one or more components in a device or system does not preclude the presence of additional components or intervening components between those components expressly identified.

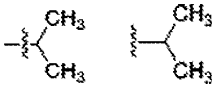
[0134] Unless otherwise stated, all crystalline forms of the compounds of the disclosure and salts thereof are also within the scope of the disclosure. The compounds of the disclosure may be isolated in various amorphous and crystalline forms, including without limitation forms which are anhydrous, hydrated, non-solvated, or solvated. Example hydrates include hemihydrates, monohydrates, dihydrates, and the like. In some embodiments, the compounds of the disclosure are anhydrous and non-solvated. By "anhydrous" is meant that the crystalline form of the compound contains essentially no bound water in the crystal lattice structure, i.e., the compound does not form a crystalline hydrate.

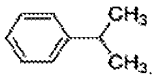
[0135] As used herein, "crystalline form" is meant to refer to a certain lattice configuration of a crystalline substance. Different crystalline forms of the same substance typically have different crystalline lattices (e.g., unit cells) which are attributed to different physical properties that are characteristic of each of the crystalline forms. In some instances, different lattice configurations have different water or solvent content. The different crystalline lattices can be identified by solid state characterization methods such as by X-ray powder diffraction (PXRD). Other characterization methods such as differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), dynamic vapor sorption (DVS), solid state NMR, and the like further help identify the crystalline form as well as help determine stability and solvent/water content.

[0136] Crystalline forms of a substance include both solvated (e.g., hydrated) and non- solvated (e.g., anhydrous) forms. A hydrated form is a crystalline form that includes water in the crystalline lattice. Hydrated forms can be stoichiometric hydrates, where the water is present in the lattice in a

certain water/molecule ratio such as for hemihydrates, monohydrates, dihydrates, etc. Hydrated forms can also be non-stoichiometric, where the water content is variable and dependent on external conditions such as humidity.

[0137] In some embodiments, the compounds of the disclosure are substantially isolated. By "substantially isolated" is meant that a particular compound is at least partially isolated from impurities. For example, in some embodiments a compound of the disclosure comprises less than about 50%, less than about 40%, less than about 30%, less than about 20%, less than about 15%, less than about 10%, less than about 5%, less than about 2.5%, less than about 1%, or less than about 0.5% of impurities. Impurities generally include anything that is not the substantially isolated compound including, for example, other crystalline forms and other substances.

[0138] Certain groups, moieties, substituents, and atoms are depicted with a wavy line. The wavy line can intersect or cap a bond or bonds. The wavy line indicates the atom through which the groups, moieties, substituents, or atoms are bonded. For example, a phenyl group that is substituted with a propyl group depicted as: 

[0139] has the following structure:  .

[0140] All amino acid abbreviations used in this disclosure are those accepted by the United States Pa-tent and Trademark Office as set forth in 37 C.F.R. § 1 .822 (B)(J).

[0141] The amino acid sequence of an antibody can be numbered using any known numbering schemes, including those described by Kabat et al., ("Kabat" numbering scheme); Al-Lazikani et al., 1997, J. Mol. Biol., 273:927-948 ("Chothia" numbering scheme); MacCallum et al., 1996, J. Mol. Biol. 262:732-745 ("Contact" numbering scheme); Lefranc et al., Dev. Comp. Immunol., 2003, 27:55-77 ("IMGT" numbering scheme); and Honegge and Pluckthun, J. Mol. Biol., 2001, 309:657-70 ("AHo" numbering scheme). Unless otherwise specified, the numbering scheme used herein is the Kabat numbering scheme. However, selection of a numbering scheme is not intended to imply differences in sequences where they do not exist, and one of skill in the art can readily confirm a sequence position by examining the amino acid sequence of one or more antibodies. Unless stated otherwise, the "EU numbering scheme" is generally used when referring to a residue in an antibody heavy chain constant region (e.g., as reported in Kabat et al., supra).

[0142] The term "glutaminyl-modified antibody" refers to an antibody with at least one covalent linkage from a glutamine side chain to a primary amine compound of the present disclosure. In particular embodiments, the primary amine compound is linked through an amide linkage on the glutamine side chain. In certain embodiments, the glutamine is an endogenous glutamine. In other embodiments, the glutamine is an endogenous glutamine made reactive by polypeptide engineering (e.g., via amino acid deletion, insertion, substitution, or mutation on the polypeptide). In additional embodiments, the glutamine is polypeptide engineered with an acyl donor glutamine-containing tag (e.g., glutamine-containing peptide tags, Q- tags or TGase recognition tag).

[0143] The term "TGase recognition tag" refers to a sequence of amino acids comprising an acceptor glutamine residue and that when incorporated into (e.g., appended to) a polypeptide sequence, under suitable conditions, is recognized by a TGase and leads to cross-linking by the TGase through a reaction between an amino acid side chain within the sequence of amino acids and a reaction partner. The recognition tag may be a peptide sequence that is not naturally present in the polypeptide comprising the TGase recognition tag. In some embodiments, the TGase recognition tag comprises at least one Gln. In some embodiments, the TGase recognition tag comprises an amino acid sequence XXQX, wherein X is any amino acid (e.g., conventional amino acid Leu, Ala, Gly, Ser, Val, Phe, Tyr, His, Arg, Asn, Glu, Asp, Cys, Gln, Ile, Met, Pro, Thr, Lys, or Trp or nonconventional amino acid). In some embodiments, the acyl donor glutamine-containing tag comprises an amino acid sequence selected from the group consisting of LLQGG (SEQ ID NO: 270), LLQG (SEQ ID NO: 271), LSLSQG (SEQ ID NO: 272), GGGLLQGG (SEQ ID NO: 273), GLLQG (SEQ ID NO: 274), LLQ, GSPLAQSHGG (SEQ ID NO: 275), GLLQGGG (SEQ ID NO: 276), GLLQGG (SEQ ID NO: 277), GLLQ (SEQ ID NO: 278), LLQLLQGA (SEQ ID NO: 279), LLQGA (SEQ ID NO: 280), LLQYQGA (SEQ ID NO: 281), LLQGSG (SEQ ID NO: 282), LLQYQG (SEQ ID NO: 283), LLQLLQG (SEQ ID NO: 284), SLLQG (SEQ ID NO: 285), LLQLQ (SEQ ID NO: 286), LLQLLQ (SEQ ID NO: 287), and LLQGR (SEQ ID NO: 288). See for example, WO2012059882, the entire contents of which are incorporated herein.

[0144] The term “aglycosylated antibody” refers to an antibody that does not comprise a glycosylation sequence that might interfere with a transglutamination reaction, for instance an antibody that does not have saccharide group at N297 on one or more heavy chains. In particular embodiments, an antibody heavy chain has an N297 mutation. In other words, the antibody is mutated to no longer have an asparagine residue at position 297 according to the EU numbering

system as disclosed by Kabat et al. In particular embodiments, an antibody heavy chain has an N297Q or an N297D mutation. Such an antibody can be prepared by site-directed mutagenesis to remove or disable a glycosylation sequence or by site-directed mutagenesis to insert a glutamine residue at site apart from any interfering glycosylation site or any other interfering structure. Such an antibody also can be isolated from natural or artificial sources. Aglycosylated antibodies also include antibodies comprising a T299 or S298P or other mutations, or combinations of mutations that result in a lack of glycosylation.

[0145] The term "deglycosylated antibody" refers to an antibody in which a saccharide group at is removed to facilitate transglutaminase-mediated conjugation. Saccharides include, but are not limited to, N-linked oligosaccharides. In some embodiments, deglycosylation is performed at residue N297. In some embodiments, removal of saccharide groups is accomplished enzymatically, included but not limited to via PNGase.

[0146] The terms "conjugated protein" or "conjugated antibody" as used herein refers to a protein or an antibody covalently linked to one or more chemical moieties. The chemical moiety can include an amine compound of the present disclosure. Linkers (**L**) and payloads (**D**) suitable for use with the present disclosure are described in detail herein. In particular embodiments, a conjugated antibody comprising a therapeutic moiety is an antibody-drug conjugate (ADC), also referred to as an antibody-payload conjugate, or an antibody-linker-payload conjugate.

[0147] The term "Drug-to-Antibody Ratio" or (DAR) is the average number of therapeutic moieties, e.g., drugs, conjugated to a binding agent of the present disclosure.

[0148] The term "Linker Antibody Ratio" or (LAR), also denoted as the lower case l in some embodiments, is the average number of reactive primary amine compounds conjugated to a binding agent of the present disclosure. Such binding agents, e.g., antibodies, can be conjugated with primary amine compounds comprising, e.g., a suitable azide or alkyne. The resulting binding agent, which is functionalized with an azide or an alkyne can subsequently react with a therapeutic moiety comprising the corresponding azide or alkyne via the 1,3-cycloaddition reaction.

[0149] The phrase "pharmaceutically acceptable amount" refers to an amount effective or sufficient in treating, reducing, alleviating, or modulating the effects or symptoms of at least one health problem in a subject in need thereof. For example, a pharmaceutically acceptable amount of an antibody or antibody-drug conjugate is an amount effective for modulating a biological target

using the antibody or antibody-drug-conjugates provided herein. Suitable pharmaceutically acceptable amounts include, but are not limited to, from about 0.001% up to about 10%, and any amount in between, such as about 0.01%, about 0.02%, about 0.03%, about 0.04%, about 0.05%, about 0.06%, about 0.07%, about 0.08%, about 0.09%, about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, or about 10% of an antibody or antibody-drug-conjugate provided herein.

[0150] The phrase "reaction pH" refers to the pH of a reaction after all reaction components or reactants have been added.

Bispecific Antigen-Binding Molecules

[0151] An anti-hCACNG1 antibody and antigen-binding fragment thereof as described herein may be monospecific, bi-specific, or multispecific. Multispecific antibodies may be specific for different epitopes of one target polypeptide or may contain antigen-binding domains specific for more than one target polypeptide. See, e.g., Tutt et al., 1991, J. Immunol. 147:60-69; Kufer *et al.*, 2004, Trends Biotechnol. 22:238-244. An anti-hCACNG1 antibody and antigen-binding fragment thereof as described herein can be linked to or co-expressed with another functional molecule, e.g., another peptide or protein. For example, an antibody or fragment thereof can be functionally linked (e.g., by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody or antibody fragment to produce a bi-specific or a multispecific antibody with a second or additional binding specificity.

[0152] Use of the expression "anti-hCACNG1 antibody" herein is intended to include both monospecific anti-hCACNG1 antibodies as well as bispecific antibodies comprising a CACNG1-binding arm and a "target"-binding arm. Thus, described herein are bispecific antibodies wherein one arm of an immunoglobulin binds human CACNG1, and the other arm of the immunoglobulin is specific for another target molecule. The CACNG1-binding arm can comprise any of the HCVR/LCVR or CDR amino acid sequences as set forth in Table 1 herein.

[0153] In certain embodiments, the CACNG1-binding arm binds to human CACNG1 and induces internalization of the CACNG1 and antibody bound thereto. In certain embodiments, the CACNG1-binding arm binds weakly to human CACNG1 and induces internalization of CACNG1 and antibody bound thereto.

[0154] In certain exemplary embodiments, the bispecific antigen-binding molecule is a bispecific antibody. Each antigen-binding domain of a bispecific antibody comprises a heavy chain variable domain (HCVR) and a light chain variable domain (LCVR). In the context of a bispecific antigen-binding molecule comprising a first and a second antigen-binding domain (*e.g.*, a bispecific antibody), the CDRs of the first antigen-binding domain may be designated with the prefix "A1" and the CDRs of the second antigen-binding domain may be designated with the prefix "A2". Thus, the CDRs of the first antigen-binding domain may be referred to herein as A1-HCDR1, A1-HCDR2, and A1-HCDR3; and the CDRs of the second antigen-binding domain may be referred to herein as A2-HCDR1, A2-HCDR2, and A2-HCDR3.

[0155] The first antigen-binding domain and the second antigen-binding domain may be directly or indirectly connected to one another to form a bispecific antigen-binding molecule as described herein. Alternatively, the first antigen-binding domain and the second antigen-binding domain may each be connected to a separate multimerizing domain. The association of one multimerizing domain with another multimerizing domain facilitates the association between the two antigen-binding domains, thereby forming a bispecific antigen-binding molecule. A "multimerizing domain" is any macromolecule, protein, polypeptide, peptide, or amino acid that has the ability to associate with a second multimerizing domain of the same or similar structure or constitution. For example, a multimerizing domain may be a polypeptide comprising an immunoglobulin C_H3 domain. A non-limiting example of a multimerizing component is an Fc portion of an immunoglobulin (comprising a C_H2-C_H3 domain), *e.g.*, an Fc domain of an IgG selected from the isotypes IgG1, IgG2, IgG3, and IgG4, as well as any allotype within each isotype group.

[0156] Bispecific antigen-binding molecules as described herein will typically comprise two multimerizing domains, *e.g.*, two Fc domains that are each individually part of a separate antibody heavy chain. The first and second multimerizing domains may be of the same IgG isotype such as, *e.g.*, IgG1/IgG1, IgG2/IgG2, IgG4/IgG4. Alternatively, the first and second multimerizing domains may be of different IgG isotypes such as, *e.g.*, IgG1/IgG2, IgG1/IgG4, IgG2/IgG4, etc.

[0157] In certain embodiments, the multimerizing domain is an Fc fragment or an amino acid sequence of from 1 to about 200 amino acids in length containing at least one cysteine residue. In other embodiments, the multimerizing domain is a cysteine residue, or a short cysteine-containing

peptide. Other multimerizing domains include peptides or polypeptides comprising or consisting of a leucine zipper, a helix-loop motif, or a coiled-coil motif.

[0158] Any bispecific antibody format or technology may be used to make the bispecific antigen-binding molecules as described herein. For example, an antibody or fragment thereof having a first antigen binding specificity can be functionally linked (e.g., by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody or antibody fragment having a second antigen-binding specificity to produce a bispecific antigen-binding molecule. Specific exemplary bispecific formats include, without limitation, *e.g.*, scFv-based or diabody bispecific formats, IgG-scFv fusions, dual variable domain (DVD)-Ig, Quadroma, knobs-into-holes, common light chain (*e.g.*, common light chain with knobs-into-holes, etc.), CrossMab, CrossFab, (SEED)body, leucine zipper, Duobody, IgG1/IgG2, dual acting Fab (DAF)-IgG, and Mab^2 bispecific formats (*see*, *e.g.*, Klein *et al.* 2012, mAbs 4:6, 1-11, and references cited therein, for a review of the foregoing formats).

[0159] In the context of bispecific antigen-binding molecules as described herein, the multimerizing domains, *e.g.*, Fc domains, may comprise one or more amino acid changes (*e.g.*, insertions, deletions or substitutions) as compared to the wild-type, naturally occurring version of the Fc domain. For example, bispecific antigen-binding molecules may comprise one or more modifications in the Fc domain that results in a modified Fc domain having a modified binding interaction (*e.g.*, enhanced or diminished) between Fc and FcRn. In one embodiment, the bispecific antigen-binding molecule comprises a modification in a C_H2 or a C_H3 region, wherein the modification increases the affinity of the Fc domain to FcRn in an acidic environment (*e.g.*, in an endosome where pH ranges from about 5.5 to about 6.0). Non-limiting examples of such Fc modifications include, *e.g.*, a modification at position 250 (*e.g.*, E or Q); 250 and 428 (*e.g.*, L or F); 252 (*e.g.*, L/Y/F/W or T), 254 (*e.g.*, S or T), and 256 (*e.g.*, S/R/Q/E/D or T); or a modification at position 428 and/or 433 (*e.g.*, L/R/S/P/Q or K) and/or 434 (*e.g.*, H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.*, 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.*, M428L) and 434S (*e.g.*, N434S) modification; a 428L, 259I (*e.g.*, V259I), and 308F (*e.g.*, V308F) modification; a 433K (*e.g.*, H433K) and a 434 (*e.g.*, 434Y) modification; a 252, 254, and 256 (*e.g.*, 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.*, T250Q and M428L); and a 307 and/or 308 modification (*e.g.*, 308F or 308P).

[0160] Also described herein are bispecific antigen-binding molecules comprising a first C_H3 domain and a second Ig C_H3 domain, wherein the first and second Ig C_H3 domains differ from one another by at least one amino acid, and wherein at least one amino acid difference reduces binding of the bispecific antibody to Protein A as compared to a bi-specific antibody lacking the amino acid difference. In one embodiment, the first Ig C_H3 domain binds Protein A and the second Ig C_H3 domain contains a mutation that reduces or abolishes Protein A binding such as an H95R modification (by IMGT exon numbering; H435R by EU numbering). The second C_H3 may further comprise a Y96F modification (by IMGT; Y436F by EU). See, for example, US Patent No. 8,586,713. Further modifications that may be found within the second C_H3 include: D16E, L18M, N44S, K52N, V57M, and V82I (by IMGT; D356E, L358M, N384S, K392N, V397M, and V422I by EU) in the case of IgG1 antibodies; N44S, K52N, and V82I (IMGT; N384S, K392N, and V422I by EU) in the case of IgG2 antibodies; and Q15R, N44S, K52N, V57M, R69K, E79Q, and V82I (by IMGT; Q355R, N384S, K392N, V397M, R409K, E419Q, and V422I by EU) in the case of IgG4 antibodies.

[0161] In certain embodiments, the Fc domain may be chimeric, combining Fc sequences derived from more than one immunoglobulin isotype. For example, a chimeric Fc domain can comprise part or all of a C_H2 sequence derived from a human IgG1, human IgG2 or human IgG4 C_H2 region, and part or all of a C_H3 sequence derived from a human IgG1, human IgG2 or human IgG4. A chimeric Fc domain can also contain a chimeric hinge region. For example, a chimeric hinge may comprise an "upper hinge" sequence, derived from a human IgG1, a human IgG2 or a human IgG4 hinge region, combined with a "lower hinge" sequence, derived from a human IgG1, a human IgG2 or a human IgG4 hinge region. A particular example of a chimeric Fc domain that can be included in any of the antigen-binding molecules set forth herein comprises, from N- to C-terminus: [IgG4 C_H1] - [IgG4 upper hinge] - [IgG2 lower hinge] - [IgG4 CH2] - [IgG4 CH3]. Another example of a chimeric Fc domain that can be included in any of the antigen-binding molecules set forth herein comprises, from N- to C-terminus: [IgG1 C_H1] - [IgG1 upper hinge] - [IgG2 lower hinge] - [IgG4 CH2] - [IgG1 CH3]. These and other examples of chimeric Fc domains that can be included in any of the antigen-binding molecules as described herein are described in US Publication 2014/0243504, published August 28, 2014, which is herein incorporated in its entirety. Chimeric Fc domains having these general structural arrangements, and variants thereof, can have altered Fc receptor binding, which in turn affects Fc effector function.

[0162] In certain embodiments, an antibody heavy chain as described herein comprises a heavy chain constant region (CH) region that comprises an amino acid sequence at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to any one of SEQ ID NO: 243, SEQ ID NO: 244, SEQ ID NO: 245, SEQ ID NO: 246, SEQ ID NO: 247, SEQ ID NO: 248, SEQ ID NO: 249, SEQ ID NO: 250, SEQ ID NO: 251, SEQ ID NO: 252, SEQ ID NO: 253, SEQ ID NO: 254, SEQ ID NO: 255, SEQ ID NO: 330. In some embodiments, the heavy chain constant region (CH) region comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 243, SEQ ID NO: 244, SEQ ID NO: 245, SEQ ID NO: 246, SEQ ID NO: 247, SEQ ID NO: 248, SEQ ID NO: 249, SEQ ID NO: 250, SEQ ID NO: 251, SEQ ID NO: 252, SEQ ID NO: 253, SEQ ID NO: 254, SEQ ID NO: 255, or SEQ ID NO: 330.

[0163] In some embodiments, an antibody heavy chain as described herein comprises an Fc domain that comprises an amino acid sequence at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to any one of SEQ ID NO: 256, SEQ ID NO: 257, SEQ ID NO: 258, SEQ ID NO: 259, SEQ ID NO: 260, SEQ ID NO: 261, SEQ ID NO: 262, SEQ ID NO: 263, SEQ ID NO: 264, SEQ ID NO: 265, SEQ ID NO: 266, or SEQ ID NO: 267. In some embodiments, the Fc domain comprises an amino acid sequence selected form the group consisting of SEQ ID NO: 256, SEQ ID NO: 257, SEQ ID NO: 258, SEQ ID NO: 259, SEQ ID NO: 260, SEQ ID NO: 261, SEQ ID NO: 262, SEQ ID NO: 263, SEQ ID NO: 264, SEQ ID NO: 265, SEQ ID NO: 266, or SEQ ID NO: 267.

Germline Mutations

[0164] The anti-hCACNG1 antibodies disclosed herein may comprise one or more amino acid substitutions, insertions and/or deletions in the framework and/or CDR regions of the heavy chain variable domains as compared to the corresponding germline sequences from which the antibodies were derived.

[0165] An anti-hCACNG1 antibody and antigen-binding fragment thereof as disclosed herein may be derived from any of the amino acid sequences disclosed herein, wherein one or more amino acids within one or more framework and/or CDR regions are mutated to the corresponding residue(s) of the germline sequence from which the antibody was derived, or to the corresponding residue(s) of another human germline sequence, or to a conservative amino acid substitution of the corresponding germline residue(s) (such sequence changes are referred to herein collectively as

"germline mutations"), and having weak or no detectable binding to a CACNG1 antigen. Several such exemplary antibodies that recognize CACNG1 are described in Table 1 herein.

[0166] Furthermore, an anti-hCACNG1 antibody and antigen-binding fragment thereof as disclosed herein may contain any combination of two or more germline mutations within the framework and/or CDR regions, *e.g.*, wherein certain individual residues are mutated to the corresponding residue of a particular germline sequence while certain other residues that differ from the original germline sequence are maintained or are mutated to the corresponding residue of a different germline sequence. Once obtained, an antibody or antigen-binding fragment that contains one or more germline mutations can be tested for one or more desired properties such as, improved binding specificity, weak or reduced binding affinity, improved or enhanced pharmacokinetic properties, reduced immunogenicity, etc. In some embodiments, an antibody or antigen-binding fragment as described herein is obtained in this general manner.

[0167] Also described herein are anti-hCACNG1 antibodies and antigen-binding fragments thereof comprising variants of any of the HCVR, LCVR, and/or CDR amino acid sequences disclosed herein having one or more conservative substitutions. For example, an anti-hCACNG1 antibody or antigen-binding fragment thereof as described herein may comprise HCVR, LCVR, and/or CDR amino acid sequences with, *e.g.*, 10 or fewer, 8 or fewer, 6 or fewer, 4 or fewer, etc. conservative amino acid substitutions relative to any of the HCVR, LCVR, and/or CDR amino acid sequences set forth in Table 1 herein. An antibody and antigen-binding fragment thereof as described herein may comprise one or more amino acid substitutions, insertions and/or deletions in the framework and/or CDR regions of the heavy and light chain variable domains as compared to the corresponding germline sequences from which the individual antigen-binding domains were derived, while maintaining or improving the desired weak-to-no detectable binding to, e.g., CACNG1. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein, *i.e.* the amino acid substitution maintains or improves the desired weak to no detectable binding affinity in the case of anti-hCACNG1 binding molecules. Examples of groups of amino acids that have side chains with similar chemical properties include (1) aliphatic side chains: glycine, alanine, valine, leucine and isoleucine; (2) aliphatic-hydroxyl side chains: serine and threonine; (3) amide-containing side

chains: asparagine and glutamine; (4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; (5) basic side chains: lysine, arginine, and histidine; (6) acidic side chains: aspartate and glutamate, and (7) sulfur-containing side chains are cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine. Alternatively, a conservative replacement is any change having a positive value in the PAM250 log-likelihood matrix disclosed in Gonnet *et al.* (1992) Science 256: 1443-1445. A "moderately conservative" replacement is any change having a nonnegative value in the PAM250 log-likelihood matrix.

[0168] Also described herein are anti-hCACNG1 antibodies and antigen-binding fragments thereof comprising an antigen-binding domain with an HCVR and/or CDR amino acid sequence that is substantially identical to any of the HCVR and/or CDR amino acid sequences disclosed herein, while maintaining or improving the desired weak affinity to CACNG1 antigen. The term "substantial identity" or "substantially identical," when referring to an amino acid sequence means that two amino acid sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 95% sequence identity, even more preferably at least 98% or 99% sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of similarity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. *See, e.g.*, Pearson (1994) Methods Mol. Biol. 24: 307-331.

[0169] Sequence similarity for polypeptides, which is also referred to as sequence identity, is typically measured using sequence analysis software. Protein analysis software matches similar sequences using measures of similarity assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, GCG software contains programs such as Gap and Bestfit which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species of organisms or between a wild type protein and a mutein thereof. *See, e.g.*, GCG Version 6.1. Polypeptide sequences also can be compared using FASTA using default or recommended parameters, a program in GCG Version 6.1. FASTA (e.g., FASTA2 and FASTA3) provides alignments and percent sequence identity of the regions of the best overlap

between the query and search sequences (Pearson (2000) supra). Another preferred algorithm when comparing a sequence as described herein to a database containing a large number of sequences from different organisms is the computer program BLAST, especially BLASTP or TBLASTN, using default parameters. *See, e.g.*, Altschul *et al.* (1990) J. Mol. Biol. 215:403-410 and Altschul *et al.* (1997) Nucleic Acids Res. 25:3389-402.

[0170] Once obtained, antigen-binding domains that contain one or more germline mutations were tested for decreased binding affinity utilizing one or more *in vitro* assays. Generally antibodies that recognize a particular antigen are typically screened for their purpose by testing for high (*i.e.* strong) binding affinity to the antigen.

[0171] Unexpected benefits, for example, improved pharmacokinetic properties and low toxicity to the patient may be realized from further modifying the antibodies as described herein by the methods described herein.

Binding Properties of the Antibodies

[0172] The term "binding" in the context of the binding of an antibody, immunoglobulin, antibody-binding fragment, or Fc-containing protein to either, *e.g.*, a predetermined antigen, such as a cell surface protein or fragment thereof, typically refers to an interaction or association between a minimum of two entities or molecular structures, such as an antibody-antigen interaction.

[0173] For instance, binding affinity typically corresponds to a K_D value of about 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less when determined by, for instance, surface plasmon resonance (SPR) technology in a BIAcore 3000 instrument using the antigen as the ligand and the antibody, Ig, antibody-binding fragment, or Fc-containing protein as the analyte (or antiligand). Cell-based binding strategies, such as fluorescent-activated cell sorting (FACS) binding assays, are also routinely used and provide binding characterization data with respect to cell-surface expressed proteins. FACS data correlates well with other methods such as radioligand competition binding and SPR (Benedict, CA, *J Immunol Methods*. 1997, 201(2):223-31; Geuijen, CA, et al. *J Immunol Methods*. 2005, 302(1-2):68-77).

[0174] Accordingly, an anti-hCACNG1 antibody and antigen-binding fragment thereof as described herein bind to the predetermined antigen or cell surface molecule (receptor) having an affinity corresponding to a K_D value that is at least ten-fold lower than its affinity for binding to a

non-specific antigen (e.g., BSA, casein). The affinity of an antibody corresponding to a K_D value that is equal to or less than ten-fold lower than a non-specific antigen may be considered non-detectable binding, however such an antibody may be paired with a second antigen binding arm for the production of a bispecific antibody as described herein.

[0175] The term "K_D" or "KD" in molar (M) refers to the dissociation equilibrium constant of a particular antibody-antigen interaction, or the dissociation equilibrium constant of an antibody or antibody-binding fragment binding to an antigen. There is an inverse relationship between K_D and binding affinity, therefore the smaller the K_D value, the higher, *i.e.* stronger, the affinity. Thus, the terms "higher affinity" or "stronger affinity" relate to a higher ability to form an interaction and therefore a smaller K_D value, and conversely the terms "lower affinity" or "weaker affinity" relate to a lower ability to form an interaction and therefore a larger K_D value. In some circumstances, a higher binding affinity (or K_D) of a particular molecule (*e.g.* antibody) to its interactive partner molecule (*e.g.* antigen X) compared to the binding affinity of the molecule (*e.g.* antibody) to another interactive partner molecule (*e.g.* antigen Y) may be expressed as a binding ratio determined by dividing the larger K_D value (lower, or weaker, affinity) by the smaller K_D (higher, or stronger, affinity), for example expressed as 5-fold or 10-fold greater binding affinity, as the case may be.

[0176] The term "k_d" (sec -1 or 1/s) refers to the dissociation rate constant of a particular antibody-antigen interaction, or the dissociation rate constant of an antibody or antibody-binding fragment. Said value is also referred to as the k_{off} value.

[0177] The term "k_a" (M-1 x sec-1 or 1/M) refers to the association rate constant of a particular antibody-antigen interaction, or the association rate constant of an antibody or antibody-binding fragment.

[0178] The term "K_A" (M-1 or 1/M) refers to the association equilibrium constant of a particular antibody-antigen interaction, or the association equilibrium constant of an antibody or antibody-binding fragment. The association equilibrium constant is obtained by dividing the k_a by the k_d.

[0179] The term "EC50" or "EC_{50}" refers to the half maximal effective concentration, which includes the concentration of an antibody which induces a response halfway between the baseline and maximum after a specified exposure time. The EC_{50} essentially represents the concentration of an antibody where 50% of its maximal effect is observed. In certain embodiments, the EC_{50} value

equals the concentration of an antibody as described herein that gives half-maximal binding to cells expressing CACNG1, as determined by *e.g.* a FACS binding assay or an androgen receptor activation luciferase assay. Thus, reduced or weaker binding is observed with an increased EC_{50}, or half maximal effective concentration value.

[0180] In one embodiment, decreased binding can be defined as an increased EC_{50} antibody concentration which enables binding to the half-maximal amount of target cells.

Sequence Variants

[0181] An anti-hCACNG1 antibody and antigen-binding fragment as described herein may comprise one or more amino acid substitutions, insertions and/or deletions in the framework and/or CDR regions of the heavy and light chain variable domains as compared to the corresponding germline sequences from which the individual antigen-binding domains were derived. Such mutations can be readily ascertained by comparing the amino acid sequences disclosed herein to germline sequences available from, for example, public antibody sequence databases. The antigen-binding molecules as described herein may comprise antigen-binding domains which are derived from any of the exemplary amino acid sequences disclosed herein, wherein one or more amino acids within one or more framework and/or CDR regions are mutated to the corresponding residue(s) of the germline sequence from which the antibody was derived, or to the corresponding residue(s) of another human germline sequence, or to a conservative amino acid substitution of the corresponding germline residue(s) (such sequence changes are referred to herein collectively as "germline mutations"). A person of ordinary skill in the art, starting with the heavy and light chain variable region sequences disclosed herein, can easily produce numerous antibodies and antigen-binding fragments which comprise one or more individual germline mutations or combinations thereof. In certain embodiments, all of the framework and/or CDR residues within the V_H and/or V_L domains are mutated back to the residues found in the original germline sequence from which the antigen-binding domain was originally derived. In other embodiments, only certain residues are mutated back to the original germline sequence, *e.g.*, only the mutated residues found within the first 8 amino acids of FR1 or within the last 8 amino acids of FR4, or only the mutated residues found within CDR1, CDR2 or CDR3. In other embodiments, one or more of the framework and/or CDR residue(s) are mutated to the corresponding residue(s) of a different germline sequence (*i.e.*, a germline sequence that is different from the germline sequence from which the antigen-binding

domain was originally derived). Furthermore, the antigen-binding domains may contain any combination of two or more germline mutations within the framework and/or CDR regions, *e.g.*, wherein certain individual residues are mutated to the corresponding residue of a particular germline sequence while certain other residues that differ from the original germline sequence are maintained or are mutated to the corresponding residue of a different germline sequence. Once obtained, antigen-binding domains that contain one or more germline mutations can be easily tested for one or more desired property such as, improved binding specificity, increased binding affinity, improved or enhanced antagonistic or agonistic biological properties (as the case may be), reduced immunogenicity, etc. Described herein are antigen-binding molecules comprising one or more antigen-binding domains obtained in this general manner.

[0182] Also described herein are antigen-binding molecules wherein one or both antigen-binding domains comprise variants of any of the HCVR, LCVR, and/or CDR amino acid sequences disclosed herein having one or more conservative substitutions. For example, antigen-binding molecules as described herein may comprise an antigen-binding domain having HCVR, LCVR, and/or CDR amino acid sequences with, *e.g.*, 10 or fewer, 8 or fewer, 6 or fewer, 4 or fewer, etc. conservative amino acid substitutions relative to any of the HCVR, LCVR, and/or CDR amino acid sequences disclosed herein. A "conservative amino acid substitution" is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (e.g., charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. Examples of groups of amino acids that have side chains with similar chemical properties include (1) aliphatic side chains: glycine, alanine, valine, leucine and isoleucine; (2) aliphatic-hydroxyl side chains: serine and threonine; (3) amide-containing side chains: asparagine and glutamine; (4) aromatic side chains: phenylalanine, tyrosine, and tryptophan; (5) basic side chains: lysine, arginine, and histidine; (6) acidic side chains: aspartate and glutamate, and (7) sulfur-containing side chains are cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, glutamate-aspartate, and asparagine-glutamine. Alternatively, a conservative replacement is any change having a positive value in the PAM250 log-likelihood matrix disclosed in Gonnet *et al.* (1992) Science 256: 1443-1445, herein incorporated by reference. A "moderately conservative" replacement is any change having a nonnegative value in the PAM250 log-likelihood matrix.

[0183] Antigen-binding molecules as described herein may comprise an antigen-binding domain with an HCVR, LCVR, and/or CDR amino acid sequence that is substantially identical to any of the HCVR, LCVR, and/or CDR amino acid sequences disclosed herein. The term "substantial identity" or "substantially identical," when referring to an amino acid sequence means that two amino acid sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 95% sequence identity, even more preferably at least 98% or 99% sequence identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of similarity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well-known to those of skill in the art. *See, e.g.*, Pearson (1994) Methods Mol. Biol. 24: 307-331, herein incorporated by reference.

[0184] Sequence similarity for polypeptides, which is also referred to as sequence identity, is typically measured using sequence analysis software. Protein analysis software matches similar sequences using measures of similarity assigned to various substitutions, deletions and other modifications, including conservative amino acid substitutions. For instance, GCG software contains programs such as Gap and Bestfit which can be used with default parameters to determine sequence homology or sequence identity between closely related polypeptides, such as homologous polypeptides from different species of organisms or between a wild type protein and a mutein thereof. *See, e.g.*, GCG Version 6.1. Polypeptide sequences also can be compared using FASTA using default or recommended parameters, a program in GCG Version 6.1. FASTA (e.g., FASTA2 and FASTA3) provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences (Pearson (2000) supra). Another preferred algorithm when comparing a sequence to a database containing a large number of sequences from different organisms is the computer program BLAST, especially BLASTP or TBLASTN, using default parameters. *See, e.g.*, Altschul *et al.* (1990) J. Mol. Biol. 215:403-410 and Altschul *et al.* (1997) Nucleic Acids Res. 25:3389-402, each herein incorporated by reference.

pH-Dependent Binding

[0185] Also described herein are anti-hCACNG1 antibodies and antigen-binding fragments thereof with pH-dependent binding characteristics. For example, an anti-hCACNG1 as described herein may exhibit reduced binding to CACNG1 at acidic pH as compared to neutral pH. Alternatively,

anti-hCACNG1 antibodies as described herein may exhibit enhanced binding to CACNG1 at acidic pH as compared to neutral pH. The expression "acidic pH" includes pH values less than about 6.2, *e.g.*, about 6.0, 5.95, 5,9, 5.85, 5.8, 5.75, 5.7, 5.65, 5.6, 5.55, 5.5, 5.45, 5.4, 5.35, 5.3, 5.25, 5.2, 5.15, 5.1, 5.05, 5.0, or less. The expression "neutral pH" means a pH of about 7.0 to about 7.4. The expression "neutral pH" includes pH values of about 7.0, 7.05, 7.1, 7.15, 7.2, 7.25, 7.3, 7.35, and 7.4.

[0186] In certain instances, "reduced binding ... at acidic pH as compared to neutral pH" is expressed in terms of a ratio of the K_D value of the antibody binding to its antigen at acidic pH to the K_D value of the antibody binding to its antigen at neutral pH (or vice versa). For example, an antibody or antigen-binding fragment thereof may be regarded as exhibiting "reduced binding to CACNG1 at acidic pH as compared to neutral pH" for purposes of the description herein if the antibody or antigen-binding fragment thereof exhibits an acidic/neutral K_D ratio of about 3.0 or greater. In certain exemplary embodiments, the acidic/neutral K_D ratio for an antibody or antigen-binding fragment as described herein can be about 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5, 13.0, 13.5, 14.0, 14.5, 15.0, 20.0. 25.0, 30.0, 40.0, 50.0, 60.0, 70.0, 100.0 or greater.

[0187] Antibodies with pH-dependent binding characteristics may be obtained, *e.g.*, by screening a population of antibodies for reduced (or enhanced) binding to a particular antigen at acidic pH as compared to neutral pH. Additionally, modifications of the antigen-binding domain at the amino acid level may yield antibodies with pH-dependent characteristics. For example, by substituting one or more amino acids of an antigen-binding domain (*e.g.*, within a CDR) with a histidine residue, an antibody with reduced antigen-binding at acidic pH relative to neutral pH may be obtained.

Antibodies Comprising Fc Variants

[0188] In some embodiments, an anti-hCACNG1 antibody and antigen-binding fragment thereof (including a multispecific antigen-binding molecule and a multidomain therapeutic protein comprising an anti-hCACNG1 antibody or an antigen-binding fragment thereof) is provided comprising an Fc domain comprising one or more mutations which enhance or diminish antibody binding to the FcRn receptor, *e.g.*, at acidic pH as compared to neutral pH. For example, antibodies as described herein may comprise a mutation in the C_H2 or a C_H3 region of the Fc domain, wherein

the mutation(s) increases the affinity of the Fc domain to FcRn in an acidic environment (*e.g.,* in an endosome where pH ranges from about 5.5 to about 6.0). Such mutations may result in an increase in serum half-life of the antibody when administered to an animal. Non-limiting examples of such Fc modifications include, *e.g.*, a modification at position 250 (e.g., E or Q); 250 and 428 (e.g., L or F); 252 (e.g., L/Y/F/W or T), 254 (e.g., S or T), and 256 (e.g., S/R/Q/E/D or T); or a modification at position 428 and/or 433 (e.g., H/L/R/S/P/Q or K) and/or 434 (*e.g.*, H/F or Y); or a modification at position 250 and/or 428; or a modification at position 307 or 308 (*e.g.*, 308F, V308F), and 434. In one embodiment, the modification comprises a 428L (*e.g.,* M428L) and 434S (*e.g.,* N434S) modification; a 428L, 259I (*e.g.,* V259I), and 308F (*e.g.,* V308F) modification; a 433K (*e.g.,* H433K) and a 434 (*e.g.,* 434Y) modification; a 252, 254, and 256 (*e.g.,* 252Y, 254T, and 256E) modification; a 250Q and 428L modification (*e.g.,* T250Q and M428L); and a 307 and/or 308 modification (*e.g.,* 308F or 308P).

[0189] For example, an anti-hCACNG1 antibody and antigen-binding fragment as described herein may comprise an Fc domain comprising one or more pairs or groups of mutations selected from the group consisting of: 250Q and 248L (*e.g.*, T250Q and M248L); 252Y, 254T and 256E (*e.g.*, M252Y, S254T and T256E); 428L and 434S (*e.g.*, M428L and N434S); and 433K and 434F (*e.g.*, H433K and N434F). All possible combinations of the foregoing Fc domain mutations, and other mutations within the antibody variable domains disclosed herein, are contemplated within the description herein.

Biological Characteristics of the Antibodies and Bispecific Antigen-Binding Molecules

[0190] Also described herein is an antibody and antigen-binding fragment thereof that binds human CACNG1 with high, medium or low affinity, depending on the therapeutic context and particular targeting properties that are desired. For example, in the context of a bispecific antigen-binding molecule, wherein one arm binds CACNG1 and another arm binds a target antigen (*e.g.*, a tumor associated antigen), it may be desirable for the target antigen-binding arm to bind the target antigen with high affinity while the anti-hCACNG1 arm binds CACNG1 with only moderate or low affinity. In this manner, preferential targeting of the antigen-binding molecule to cells expressing the target antigen may be achieved while avoiding general/untargeted CACNG1 binding and the consequent adverse side effects associated therewith.

[0191] Also described herein are antibodies, antigen-binding fragments, and bispecific antibodies thereof that bind human CACNG1 with weak (*i.e.* low) or even no detectable affinity. In some embodiments, an antibody and antigen-binding fragment thereof as described herein binds human CACNG1 (*e.g.*, at 37°C) with a K_D of greater than about 100 nM as measured by surface plasmon resonance. In some embodiments, an antibody or antigen-binding fragment as described herein binds CACNG1 with a K_D of greater than about greater than about 110 nM, at least 120 nM, greater than about 130 nM, greater than about 140 nM, greater than about 150 nM, at least 160 nM, greater than about 170 nM, greater than about 180 nM, greater than about 190 nM, greater than about 200 nM, greater than about 250 nM, greater than about 300 nM, greater than about 400 nM, greater than about 500 nM, greater than about 600 nM, greater than about 700 nM, greater than about 800 nM, greater than about 900 nM, or greater than about 1 μM, or with no detectable affinity, as measured by surface plasmon resonance (*e.g.*, mAb-capture or antigen-capture format), or a substantially similar assay.

Epitope Mapping and Related Technologies

[0192] The epitope on CACNG1 to which an anti-hCACNG1 antibody and antigen-binding fragment thereof as described herein may consist of a single contiguous sequence of 3 or more (*e.g.*, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more) amino acids of a CACNG1 protein. Alternatively, the epitope may consist of a plurality of non-contiguous amino acids (or amino acid sequences) of CACNG1. The term "epitope" refers to an antigenic determinant that interacts with a specific antigen binding site in the variable region of an antibody molecule known as a paratope. A single antigen may have more than one epitope. Thus, different antibodies may bind to different areas on an antigen and may have different biological effects. Epitopes may be either conformational or linear. A conformational epitope is produced by spatially juxtaposed amino acids from different segments of the linear polypeptide chain. A linear epitope is one produced by adjacent amino acid residues in a polypeptide chain. In certain circumstances, an epitope may include moieties of saccharides, phosphoryl groups, or sulfonyl groups on the antigen.

[0193] Various techniques known to persons of ordinary skill in the art can be used to determine whether an antigen-binding domain of an antibody "interacts with one or more amino acids" within a polypeptide or protein. Exemplary techniques include, *e.g.*, routine cross-blocking assay such as that described Antibodies, Harlow and Lane (Cold Spring Harbor Press, Cold Spring Harb., NY), alanine scanning mutational analysis, peptide blots analysis (Reineke, 2004, Methods Mol Biol

248:443-463), and peptide cleavage analysis. In addition, methods such as epitope excision, epitope extraction and chemical modification of antigens can be employed (Tomer, 2000, Protein Science 9:487-496). Another method that can be used to identify the amino acids within a polypeptide with which an antigen-binding domain of an antibody interacts is hydrogen/deuterium exchange detected by mass spectrometry. In general terms, the hydrogen/deuterium exchange method involves deuterium-labeling the protein of interest, followed by binding the antibody to the deuterium-labeled protein. Next, the protein/antibody complex is transferred to water to allow hydrogen-deuterium exchange to occur at all residues except for the residues protected by the antibody (which remain deuterium-labeled). After dissociation of the antibody, the target protein is subjected to protease cleavage and mass spectrometry analysis, thereby revealing the deuterium-labeled residues which correspond to the specific amino acids with which the antibody interacts. *See, e.g.*, Ehring (1999) *Analytical Biochemistry* 267(2):252-259; Engen and Smith (2001) *Anal. Chem. 73*:256A-265A. X-ray crystallography of the antigen/antibody complex may also be used for epitope mapping purposes.

[0194] Also described herein are anti-hCACNG1 antibodies that bind to the same epitope as any of the specific exemplary antibodies described herein (*e.g.* antibodies comprising any of the amino acid sequences as set forth in Table 1 herein). Likewise, also described herein are anti-hCACNG1 antibodies that compete for binding to CACNG1 with any of the specific exemplary antibodies described herein (*e.g.* antibodies comprising any of the amino acid sequences as set forth in Table 1 herein).

[0195] One can easily determine whether a particular antigen-binding molecule (*e.g.*, antibody) or antigen-binding domain thereof binds to the same epitope as, or competes for binding with, a reference antigen-binding molecule as described herein by using routine methods known in the art. For example, to determine if a test antibody binds to the same epitope on CACNG1 as a reference bispecific antigen-binding molecule as described herein, the reference bispecific molecule is first allowed to bind to a CACNG1 protein. Next, the ability of a test antibody to bind to the CACNG1 molecule is assessed. If the test antibody is able to bind to CACNG1 following saturation binding with the reference bispecific antigen-binding molecule, it can be concluded that the test antibody binds to a different epitope of CACNG1 than the reference bispecific antigen-binding molecule. On the other hand, if the test antibody is not able to bind to the CACNG1 molecule following saturation binding with the reference bispecific antigen-binding molecule, then the test antibody

may bind to the same epitope of CACNG1 as the epitope bound by the reference bispecific antigen-binding molecule as described herein. Additional routine experimentation (*e.g.*, peptide mutation and binding analyses) can then be carried out to confirm whether the observed lack of binding of the test antibody is in fact due to binding to the same epitope as the reference bispecific antigen-binding molecule or if steric blocking (or another phenomenon) is responsible for the lack of observed binding. Experiments of this sort can be performed using ELISA, RIA, Biacore, flow cytometry or any other quantitative or qualitative antibody-binding assay available in the art. In accordance with some embodiments described herein, two antigen-binding proteins bind to the same (or overlapping) epitope if, *e.g.*, a 1-, 5-, 10-, 20- or 100-fold excess of one antigen-binding protein inhibits binding of the other by at least 50% but preferably 75%, 90% or even 99% as measured in a competitive binding assay (see, e.g., Junghans et al., Cancer Res. 1990:50:1495-1502). Alternatively, two antigen-binding proteins are deemed to bind to the same epitope if essentially all amino acid mutations in the antigen that reduce or eliminate binding of one antigen-binding protein reduce or eliminate binding of the other. Two antigen-binding proteins are deemed to have "overlapping epitopes" if only a subset of the amino acid mutations that reduce or eliminate binding of one antigen-binding protein reduce or eliminate binding of the other.

[0196] To determine if an antibody or antigen-binding domain thereof competes for binding with a reference antigen-binding molecule, the above-described binding methodology is performed in two orientations: In a first orientation, the reference antigen-binding molecule is allowed to bind to a CACNG1 protein under saturating conditions followed by assessment of binding of the test antibody to the CACNG1 molecule. In a second orientation, the test antibody is allowed to bind to a CACNG1 molecule under saturating conditions followed by assessment of binding of the reference antigen-binding molecule to the CACNG1 molecule. If, in both orientations, only the first (saturating) antigen-binding molecule is capable of binding to the CACNG1 molecule, then it is concluded that the test antibody and the reference antigen-binding molecule compete for binding to CACNG1. As will be appreciated by a person of ordinary skill in the art, an antibody that competes for binding with a reference antigen-binding molecule may not necessarily bind to the same epitope as the reference antibody, but may sterically block binding of the reference antibody by binding an overlapping or adjacent epitope.

Preparation of Antigen-Binding Domains and Construction of Bispecific Molecules

[0197] Antigen-binding domains specific for particular antigens can be prepared by any antibody generating technology known in the art. Once obtained, two different antigen-binding domains, specific for two different antigens (*e.g.*, CACNG1 and a target antigen), can be appropriately arranged relative to one another to produce a bispecific antigen-binding molecule as described herein using routine methods. (A discussion of exemplary bispecific antibody formats that can be used to construct the bispecific antigen-binding molecules as described herein is provided elsewhere herein). In certain embodiments, one or more of the individual components (*e.g.*, heavy and light chains) of the antigen-binding molecules as described herein are derived from chimeric, humanized or fully human antibodies. Methods for making such antibodies are well known in the art. For example, one or more of the heavy and/or light chains of the antigen-binding molecules as described herein can be prepared using VELOCIMMUNE™ technology. Using VELOCIMMUNE™ technology (or any other human antibody generating technology), high affinity chimeric antibodies to a particular antigen (*e.g.*, CACNG1) are initially isolated having a human variable region and a mouse constant region. The antibodies are characterized and selected for desirable characteristics, including affinity, selectivity, epitope, etc. The mouse constant regions are replaced with a desired human constant region to generate fully human heavy and/or light chains that can be incorporated into the antigen-binding molecules as described herein.

[0198] Genetically engineered animals may be used to make human bispecific antigen-binding molecules. For example, a genetically modified mouse can be used which is incapable of rearranging and expressing an endogenous mouse immunoglobulin light chain variable sequence, wherein the mouse expresses only one or two human light chain variable domains encoded by human immunoglobulin sequences operably linked to the mouse kappa constant gene at the endogenous mouse kappa locus. Such genetically modified mice can be used to isolate heavy chain and light chain variable regions to produce fully human bispecific antigen-binding molecules. As such, the fully human bispecific antigen-binding molecules comprise two different heavy chains that associate with the same light chain. (*See*, *e.g.*, US 2011/0195454). Fully human refers to an antibody, or antigen-binding fragment or immunoglobulin domain thereof, comprising an amino acid sequence encoded by a DNA derived from a human sequence over the entire length of each polypeptide of the antibody or antigen-binding fragment or immunoglobulin domain thereof. In some instances, the fully human sequence is derived from a protein endogenous to a human. In other instances, the fully human protein or protein sequence comprises a chimeric sequence

wherein each component sequence is derived from human sequence. While not being bound by any one theory, chimeric proteins or chimeric sequences are generally designed to minimize the creation of immunogenic epitopes in the junctions of component sequences, *e.g.* compared to any wild-type human immunoglobulin regions or domains.

[0199] Bispecific antigen-binding molecules may be constructed with one heavy chain having a modified Fc domain that abrogates its binding to Protein A, thus enabling a purification method that yields a heterodimeric protein. See, for example, US Patent No. 8,586,713. As such, the bispecific antigen-binding molecules comprise a first C_H3 domain and a second Ig C_H3 domain, wherein the first and second Ig C_H3 domains differ from one another by at least one amino acid, and wherein at least one amino acid difference reduces binding of the bispecific antibody to Protein A as compared to a bi-specific antibody lacking the amino acid difference. In one embodiment, the first Ig C_H3 domain binds Protein A and the second Ig C_H3 domain contains a mutation/modification that reduces or abolishes Protein A binding such as an H95R modification (by IMGT exon numbering; H435R by EU numbering). The second C_H3 may further comprise a Y96F modification (by IMGT; Y436F by EU).

Bioequivalents

[0200] Antigen-binding molecules having amino acid sequences that vary from those of the exemplary molecules disclosed herein but that retain the ability to bind CACNG1 are also described herein. Such variant molecules may comprise one or more additions, deletions, or substitutions of amino acids when compared to parent sequence, but exhibit biological activity that is essentially equivalent to that of the described bispecific antigen-binding molecules.

[0201] Antigen-binding molecules that are bioequivalent to any of the exemplary antigen-binding molecules set forth herein are also described. Two antigen-binding proteins, or antibodies, are considered bioequivalent if, for example, they are pharmaceutical equivalents or pharmaceutical alternatives whose rate and extent of absorption do not show a significant difference when administered at the same molar dose under similar experimental conditions, either single does or multiple dose. Some antigen-binding proteins will be considered equivalents or pharmaceutical alternatives if they are equivalent in the extent of their absorption but not in their rate of absorption and yet may be considered bioequivalent because such differences in the rate of absorption are intentional and are reflected in the labeling, are not essential to the attainment of effective body

drug concentrations on, e.g., chronic use, and are considered medically insignificant for the particular drug product studied.

[0202] In one embodiment, two antigen-binding proteins are bioequivalent if there are no clinically meaningful differences in their safety, purity, and potency.

[0203] In one embodiment, two antigen-binding proteins are bioequivalent if a patient can be switched one or more times between the reference product and the biological product without an expected increase in the risk of adverse effects, including a clinically significant change in immunogenicity, or diminished effectiveness, as compared to continued therapy without such switching.

[0204] In one embodiment, two antigen-binding proteins are bioequivalent if they both act by a common mechanism or mechanisms of action for the condition or conditions of use, to the extent that such mechanisms are known.

[0205] Bioequivalence may be demonstrated by in vivo and in vitro methods. Bioequivalence measures include, e.g., (a) an in vivo test in humans or other mammals, in which the concentration of the antibody or its metabolites is measured in blood, plasma, serum, or other biological fluid as a function of time; (b) an in vitro test that has been correlated with and is reasonably predictive of human in vivo bioavailability data; (c) an in vivo test in humans or other mammals in which the appropriate acute pharmacological effect of the antibody (or its target) is measured as a function of time; and (d) in a well-controlled clinical trial that establishes safety, efficacy, or bioavailability or bioequivalence of an antigen-binding protein.

[0206] Bioequivalent variants of the exemplary bispecific antigen-binding molecules set forth herein may be constructed by, for example, making various substitutions of residues or sequences or deleting terminal or internal residues or sequences not needed for biological activity. For example, cysteine residues not essential for biological activity can be deleted or replaced with other amino acids to prevent formation of unnecessary or incorrect intramolecular disulfide bridges upon renaturation. In other contexts, bioequivalent antigen-binding proteins may include variants of the exemplary bispecific antigen-binding molecules set forth herein comprising amino acid changes which modify the glycosylation characteristics of the molecules, *e.g.*, mutations which eliminate or remove glycosylation.

Species Selectivity and Species Cross-Reactivity

[0207] In some embodiments, antigen-binding molecules as described herein bind to human CACNG1 but not to CACNG1 from other species. Also described herein are antigen-binding molecules that bind to human CACNG1 and to CACNG1 from one or more non-human species.

[0208] In some embodiments, antigen-binding molecules as described herein that bind to human CACNG1 may bind, or not bind, as the case may be, to one or more of mouse, rat, guinea pig, hamster, gerbil, pig, cat, dog, rabbit, goat, sheep, cow, horse, camel, cynomolgus, marmoset, rhesus or chimpanzee CACNG1.

Antibody-Drug Conjugates (ADCs)

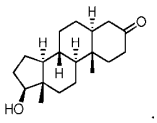
[0209] Also described herein are antibody-drug conjugates (ADCs) comprising an anti-hCACNG1 antibody or an antigen-binding fragment thereof conjugated to a drug, e.g., payload or molecular cargo, (e.g., a small molecule and/or therapeutic moiety, etc). Anti-hCACNG1 antibodies, or antigen-binding fragment thereof, conjugated to a therapeutic moiety are also provided. In general terms, the ADCs comprise: $A - [L - P]_y$, in which A is an antigen-binding molecule, *e.g.* an anti-hCACNG1 antibody, or a fragment thereof (*e.g.*, a fragment comprising at least a HCDR3 selected from any of the HCDR3 amino acid sequences listed in Table 1), L is a linker, P is the payload or molecular cargo, and y is an integer from 1 to 30.

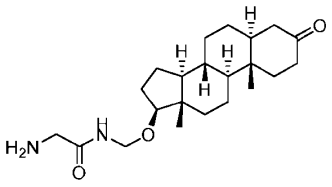
[0210] In various embodiments, the ADC comprises an anti-hCACNG1 antibody or antigen-binding fragment thereof that comprises the CDRs of a HCVR or a LCVR having the amino acid sequences of the SEQ ID NOs set forth in Table 1 (*e.g.*, SEQ ID NOs: 2, 18, 34, 50, 66, 82, 98, 114, 130, 146, 162, 178, 294, and 314 or 10, 26, 42, 58, 74, 90, 106, 122, 138, 154, 170, 186, 301 and 322), or specific HCVR/LCVR pairs (*e.g.*, SEQ ID NOs: 2/10, 18/26, 34/42, 50/58, 66/74, 82/90, 98/106, 114/122, 130/138, 146/154, 162/170, 178/186, 294/301, and 314/322). In some cases, the anti-hCACNG1 antibody or fragment comprises CDRs with the amino acid sequences of the SEQ ID NOs or sequences set forth in Table 1 (*e.g.*, SEQ ID NOs: 4-6-8-12-AAS-16, 20-22-24-26-28-ATS-32, 36-38-40-44-KAS-48, 52-54-56-60-GAS-64; 68-70-72-76-AAS-80 84-86-88-92-AAS-96; 100-102-104-108-AAS-112, 116-118-120-124-GA-128; 132-134-136-140-GAS-144; 148-150-152-156-RN-160; 164-166-168-172-DNN-176; 180-182-186-188-GAS-192; and 36-297-299-303-YNS-305; and 316-318-152-324-RNN-326). In some cases, the anti-hCACNG1 antibody or fragment comprises a HCVR and a LCVR having the amino acid sequences of the SEQ ID NOs

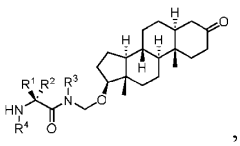
set forth in Table 1 (*e.g.*, SEQ ID NOs: 2, 18, 34, 50, 66, 82, 98, 114, 130, 146, 162, 178, 294, and 314 and 10, 26, 42, 58, 74, 90, 106, 122, 138, 154, 170, 186, 301, and 322), or specific amino acid sequence pairs (*e.g.*, SEQ ID NOs: 2/10, 18/26, 34/42, 50/58, 66/74, 82/90, 98/106, 114/122, 130/138, 146/154, 162/170, 178/186, 294/301, and 314/322).

[0211] In some embodiments, the payload or molecular cargo comprises a small molecule as a therapeutic agent, e.g. a therapeutic agent that may be useful for treating muscle wasting or genetic muscle diseases. A small molecule (SM) can enter cells easily because it has a low molecular weight (typically, up to about 1 kDa). Once inside the cells, the small molecule can affect other molecules, such as proteins, and may, for example, cause cancer cells to die. This is different from many large molecular weight molecules such as antibodies. An example, of a small molecule may be conjugated to an anti-CACNG1 antigen-binding protein, to form an anti-CACNG1:SM conjugate.

[0212] Therapeutic agents that may be useful for treating muscle wasting or genetic muscle diseases include testosterone and biologically active variants thereof, (e.g., dihydrotestosterone (DHT), or prodrugs or derivatives thereof), β2-adrenergic receptor agonists (e.g., clenbuterol), rapamycin or its analogs, MAPK inhibitors, or histone deacetylase inhibitors, etc.

[0213] In one embodiment, the therapeutic agent conjugated to the antibodies of the present disclosure is DHT having a structure  .

[0214] In one embodiment, therapeutic agent conjugated to the antibodies of the present disclosure is proDHT having a structure:  .

[0215] In one embodiment, therapeutic agent conjugated to the antibodies of the present disclosure is proDHT having a structure:  ,

wherein R_1 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

R2 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

R_3 is selected from the group consisting of H and a C_{1-3} alkyl, and

R_4 is selected from the group consisting of H and a C_{1-3} alkyl.

[0216] Also provided herein are antibody-radionuclide conjugates (ARCs) comprising anti-hCACNG1 antibodies conjugated to one or more radionuclides. Exemplary radionuclides that can be used in the context of this aspect of the disclosure include, but are not limited to, *e.g.*, ^{225}Ac, ^{212}Bi, ^{213}Bi, ^{131}I, ^{186}Re, ^{227}Th, ^{222}Rn, ^{223}Ra, ^{224}Ra, and ^{90}Y.

[0217] Linkers (L)

[0218] In certain embodiments provided herein, ADCs are provided comprising, e.g., an anti-hCACNG1 antigen-binding protein conjugated to a therapeutic agent (*e.g.*, any of the therapeutic agents disclosed above) via a linker molecule. Linkers are any group or moiety that links, connects, or bonds the antibody or antigen-binding proteins described herein with a therapeutic moiety, *e.g.* cytotoxic agent. Suitable linkers may be found, for example, in *Antibody-Drug Conjugates and Immunotoxins*; Phillips, G. L., Ed.; Springer Verlag: New York, 2013; *Antibody-Drug Conjugates*; Ducry, L., Ed.; Humana Press, 2013; *Antibody-Drug Conjugates*; Wang, J., Shen, W.-C., and Zaro, J. L., Eds.; Springer International Publishing, 2015, the contents of each incorporated herein in their entirety by reference. Generally, suitable binding agent linkers for the antibody conjugates described herein are those that are sufficiently stable to exploit the circulating half-life of the antibody and, at the same time, capable of releasing its payload after antigen-mediated internalization of the conjugate. Linkers can be cleavable or non-cleavable. Cleavable linkers include linkers that are cleaved by intracellular metabolism following internalization, *e.g.*, cleavage via hydrolysis, reduction, or enzymatic reaction. Non-cleavable linkers include linkers that release an attached payload via lysosomal degradation of the antibody following internalization. Suitable linkers include, but are not limited to, acid-labile linkers, hydrolysis-labile linkers, enzymatically cleavable linkers, reduction labile linkers, self-immolative linkers, and non-cleavable linkers. Suitable linkers also include, but are not limited to, those that are or comprise peptides,

glucuronides, succinimide-thioethers, polyethylene glycol (PEG) units, hydrazones, mal-caproyl units, dipeptide units, valine-citrulline units, and para-aminobenzyl (PAB) units.

[0219] Any linker molecule or linker technology known in the art can be used to create or construct an ADC of the present disclosure. In certain embodiments, the linker is a cleavable linker. According to other embodiments, the linker is a non-cleavable linker. Exemplary linkers that can be used in the context of the present disclosure include, linkers that comprise or consist of *e.g.*, MC (6-maleimidocaproyl), MP (maleimidopropanoyl), val-cit (valine-citrulline), val-ala (valine-alanine), val-gly (valine-glycine), dipeptide site in protease-cleavable linker, ala-phe (alanine-phenylalanine), dipeptide site in protease-cleavable linker, PAB (p-aminobenzyloxycarbonyl), SPP (N-Succinimidyl 4-(2-pyridylthio) pentanoate), SMCC (N-Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1 carboxylate), SIAB (N-Succinimidyl (4-iodo-acetyl)aminobenzoate), and variants and combinations thereof. Additional examples of linkers that can be used in the context of the present disclosure are provided, *e.g.*, in US 7,754,681 and in Ducry, Bioconjugate Chem., 2010, *21*:5-13, and the references cited therein, the contents of which are incorporated by reference herein in their entireties.

[0220] In certain embodiments, the linkers are stable in physiological conditions. In certain embodiments, the linkers are cleavable, for instance, able to release at least the payload portion in the presence of an enzyme or at a particular pH range or value. In some embodiments, a linker comprises an enzyme-cleavable moiety. Illustrative enzyme-cleavable moieties include, but are not limited to, peptide bonds, ester linkages, hydrazones, and disulfide linkages. In some embodiments, the linker comprises a cathepsin-cleavable linker.

[0221] In some embodiments, the linker comprises a non-cleavable moiety.

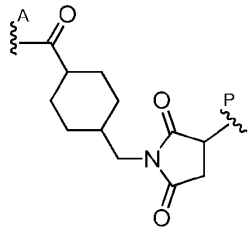
[0222] Suitable linkers also include, but are not limited to, those that are chemically bonded to two cysteine residues of a single binding agent, *e.g.*, antibody. Such linkers can serve to mimic the antibody's disulfide bonds that are disrupted as a result of the conjugation process.

[0223] In some embodiments, the linker comprises one or more amino acids. Suitable amino acids include natural, non-natural, standard, non-standard, proteinogenic, non-proteinogenic, and L- or D- α-amino acids. In some embodiments, the linker comprises alanine, valine, glycine, leucine, isoleucine, methionine, tryptophan, phenylalanine, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, or citrulline, a

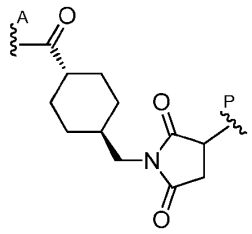
derivative thereof, or combination thereof. In certain embodiments, one or more side chains of the amino acids is linked to a side chain group, described below. In some embodiments, the linker comprises valine and citrulline. In some embodiments, the linker comprises lysine, valine, and citrulline. In some embodiments, the linker comprises lysine, valine, and alanine. In some embodiments, the linker comprises valine and alanine.

[0224] In some embodiments, the linker comprises a self-immolative group. The self-immolative group can be any such group known to those of skill. In particular embodiments, the self-immolative group is *p*-aminobenzyl (PAB), or a derivative thereof. Useful derivatives include *p*-aminobenzyloxycarbonyl (PABC). Those of skill will recognize that a self-immolative group is capable of carrying out a chemical reaction which releases the remaining atoms of a linker from a payload.

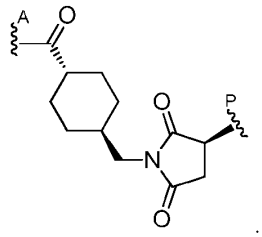
[0225] In some embodiments, the linker is:



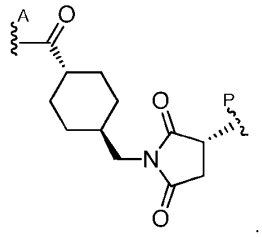
wherein $\overset{A}{\sim}$ is a bond to the antibody or antigen-binding protein (*e.g.*, via lysine residue) and $\sim\!\!\!\sim^{P}$ is a bond to the therapeutic payload (*e.g.*, testosterone or a biologically equivalent variant thereof). In some embodiments, the linker is:



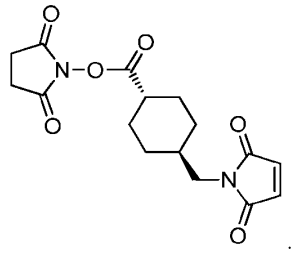
wherein $\sim\!\!\!\!\!\!\!\!\!\!\!\!\!\!^{A}$ is a bond to the antibody or antigen-binding protein (*e.g.*, via lysine residue) and $\sim\!\!\!\!\!\!\!\!\!\!\!\!\!\!^{P}$ is a bond to a therapeutic payload (*e.g.*, testosterone or a biologically equivalent variant thereof). In certain embodiments, the linker is:



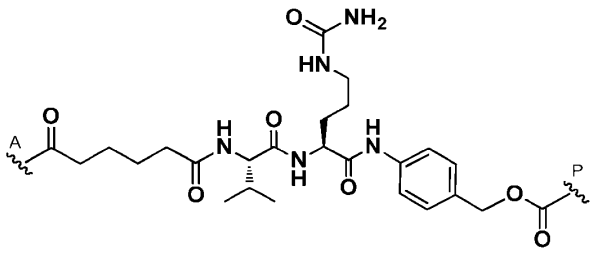
[0226] In certain embodiments, the linker is:



[0227] In some embodiments, the linker is derived from maleimidylmethyl-4-trans-cyclohexanecarboxysuccinate:

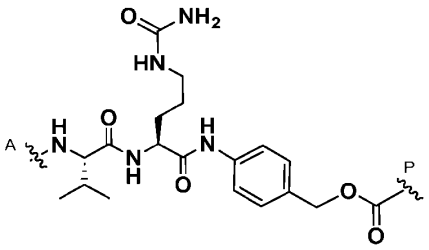


[0228] In some embodiments, the linker is:



wherein —A is a bond to the antibody or antigen-binding protein (*e.g.*, via lysine residue) and —P is a bond to therapeutic payload (*e.g.*, testosterone or a biologically equivalent variant thereof).

[0229] In some embodiments, the linker is:



wherein —A is a bond to the antibody or antigen-binding protein (*e.g.*, via lysine residue) and —P is a bond to therapeutic payload (*e.g.*, testosterone or a biologically equivalent variant thereof).

[0230] The present disclosure comprises ADCs in which a linker connects an anti-hCACNG1 antigen-binding protein as described herein to therapeutic agent through an attachment at a particular amino acid within the antibody or antigen-binding molecule. Exemplary amino acid attachments that can be used in the context of this aspect, *e.g.*, lysine (*see, e.g.*, US 5,208,020; US 2010/0129314; Hollander *et al.*, *Bioconjugate Chem.*, 2008, 19:358-361; WO 2005/089808; US 5,714,586; and US 2013/0101546), cysteine (*see, e.g.*, US 2007/0258987; WO 2013/055993; WO 2013/055990; WO 2013/053873; WO 2013/053872; WO 2011/130598; US 2013/0101546; and US 7,750,116), selenocysteine (*see, e.g.*, WO 2008/122039; and Hofer *et al.*, *Proc. Natl. Acad. Sci., USA, 2008, 105*:12451-12456), formyl glycine (*see, e.g.*, Carrico *et al.*, *Nat. Chem. Biol.*, 2007, 3:321-322; Agarwal *et al.*, *Proc. Natl. Acad. Sci., USA, 2013, 110*:46-51, and Rabuka *et al.*, *Nat.*

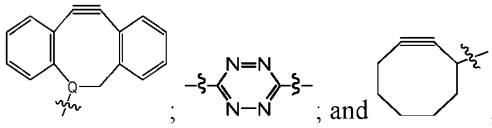
Protocols, 2012, *10*:1052-1067), non-natural amino acids (*see, e.g.*, WO 2013/068874, and WO 2012/166559), and acidic amino acids (*see, e.g.*, WO 2012/05982). Linkers can also be conjugated to an antigen-binding protein via attachment to carbohydrates (*see, e.g.*, US 2008/0305497, WO 2014/065661, and Ryan *et al.*, *Food & Agriculture Immunol.*, 2001, *13*:127-130) and disulfide linkers (*see, e.g.*, WO 2013/085925, WO 2010/010324, WO 2011/018611, and Shaunak *et al.*, *Nat. Chem. Biol.*, 2006, *2*:312-313). Site specific conjugation techniques can also be employed to direct conjugation to particular residues of the antibody or antigen binding protein (*see, e.g.*, Schumacher *et al. J Clin Immunol* (2016) 36(Suppl 1): 100). Site specific conjugation techniques, include, but are not limited to glutamine conjugation via transglutaminase (*see e.g.*, Schibli, Angew Chemie Inter Ed. 2010, 49 ,9995). In some embodiments, a residue of an antibody as described herein, e.g., a residue in a heavy chain constant region of the antibody, may be substituted with a glutamine to further facilitate glutamine conjugation via transglutaminase. As a non-limiting example, a human heavy chain constant region may be modified with the N180Q substitution found in the sequence of the human IgG1 heavy chain constant region as set forth in SEQ ID NO: 269. Such substitution provides for a total of 4 glutamines for conjugation by transglutaminase.

[0231] In one aspect of the present disclosure, the linker (L) has the following structure:

-L1-B-L2- , wherein:

L1 is a first linker unit covalently attached to the antigen-binding protein;

B is absent or a unit comprising at least one adduct of group B', where the group B' is selected from

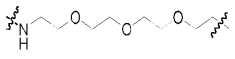


-N_3, , ; ; and , where Q is C or N;

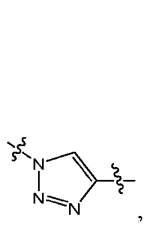
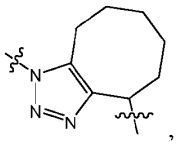
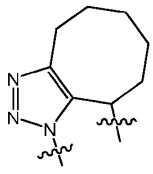
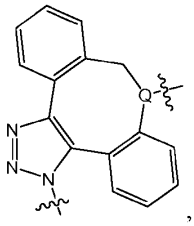
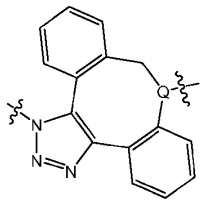
L2 is absent or a second linker unit covalently attached to the unit B via at least one group B", wherein the group B' and the group B" form the at least one adduct,

provided that when B is absent, L2 is also absent and L1 is covalently attached to the therapeutic agent, and when L2 is present, L2 is covalently attached to the therapeutic agent.

[0232] In some embodiments, L1 comprises C_{1-6} alkyl, phenyl, -NH-, -C(O)-, $-(CH_2)_u$-NH-C(O)-, $-(CH_2)_u$-C(O)-NH-, $-(CH_2-CH_2-O)_v$-, $-(CH_2)_u-(O-CH_2-CH_2)_v$-C(O)-NH-, a peptide unit comprising from 2 to 4 amino acids, or combinations thereof, each of which may be optionally substituted with one or more of -S-, $-S(O_2)$-, -C(O)-, $-C(O_2)$-; and CO_2H, wherein subscripts u and v are independently an integer from 1 to 8.

[0233] In one embodiment, L1 is  .

[0234] In some embodiments, B has a structure selected from the group consisting of:

 ,  ,  ,  , and  , wherein Q is C or N.

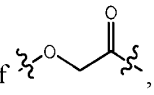
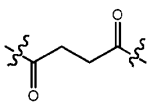
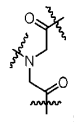
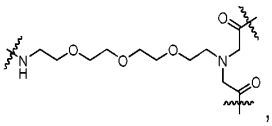
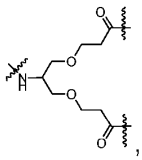
[0235] In some embodiments, L2 has a structure:

-SP1-AA-SP2- (L2), wherein:

SP1 is absent or a first spacer unit;

AA is absent or a peptide unit comprising from 2 to 4 amino acids;

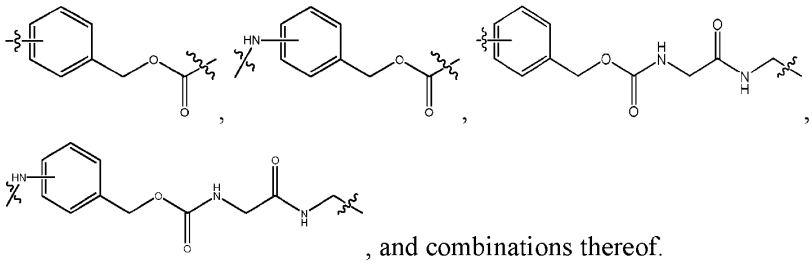
SP2 is absent or a second spacer unit covalently attached to the therapeutic agent.

[0236] In some embodiments, SP1 is absent or selected from the group consisting of  ,  ,  ,  ,  , $C_{1\text{-}6}$ alkyl, -$(CH_2$-CH_2-$O)_v$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O), -O-CH_2-C(O)-NH, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-NH, -NH-, -C(O)-, -NH-C(O)-, -NH-$(CH_2)_u$-, -NH-$(CH_2)_u$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-, -NH-$(CH_2$-CH_2-$O)_v$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-, -$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-C(O)-NH-, or combinations thereof; wherein subscripts u and v are independently an integer from 1 to 8.

[0237] In some embodiments, the AA is a peptide unit comprising from 2 to 4 amino acids selected from glycine, valine, phenylalanine, proline, glutamic acid, lysine, N,N-dipropyl lysine, phenylalanine, and citrulline, and combinations thereof.

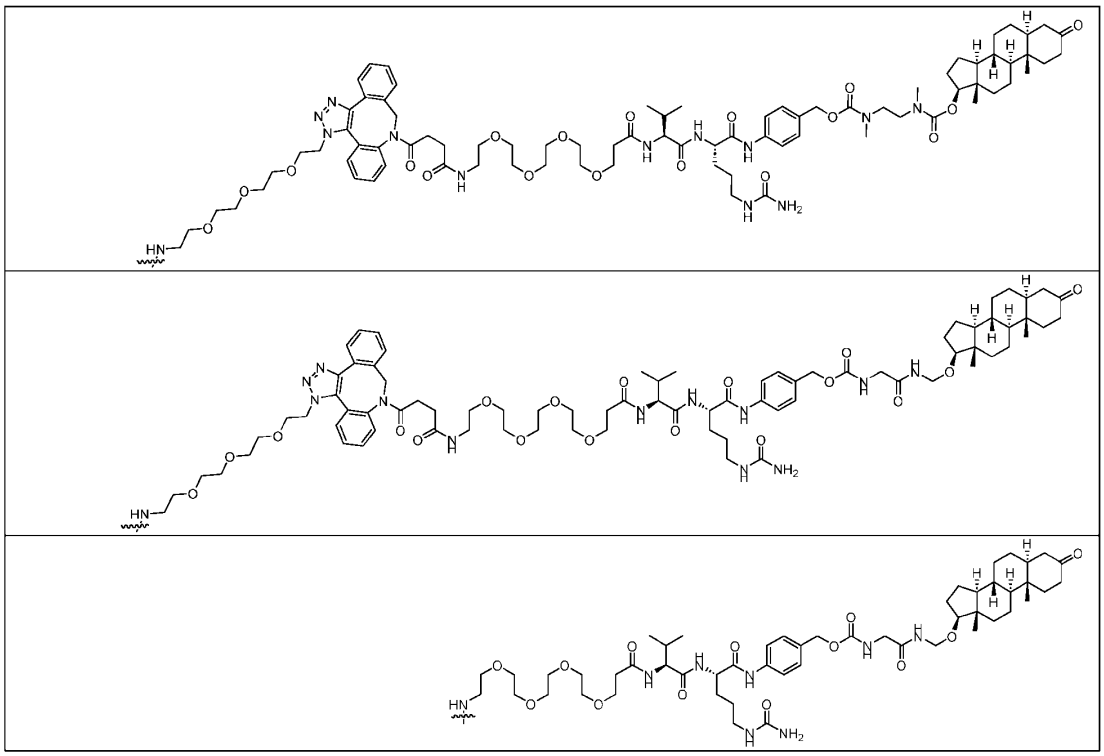
[0238] In some embodiments, the AA is valine-citrulline, valine-alanine, valine-lysine, valine-N,N-dipropyl lysine, phenylalanine-lysine, glycine-glycine-glycine (GGG), glycine-glycine-glycine-glycine (GGGG (SEQ ID NO: 289)), glycine-glycine-phenylalanine (GGF), glycine-glycine-phenylalanine-glycine (GGFG (SEQ ID NO: 290)), L-glutamic acid-valine-citrulline (^{L}EVC), and D-glutamic acid-valine-citrulline (^{D}EVC).

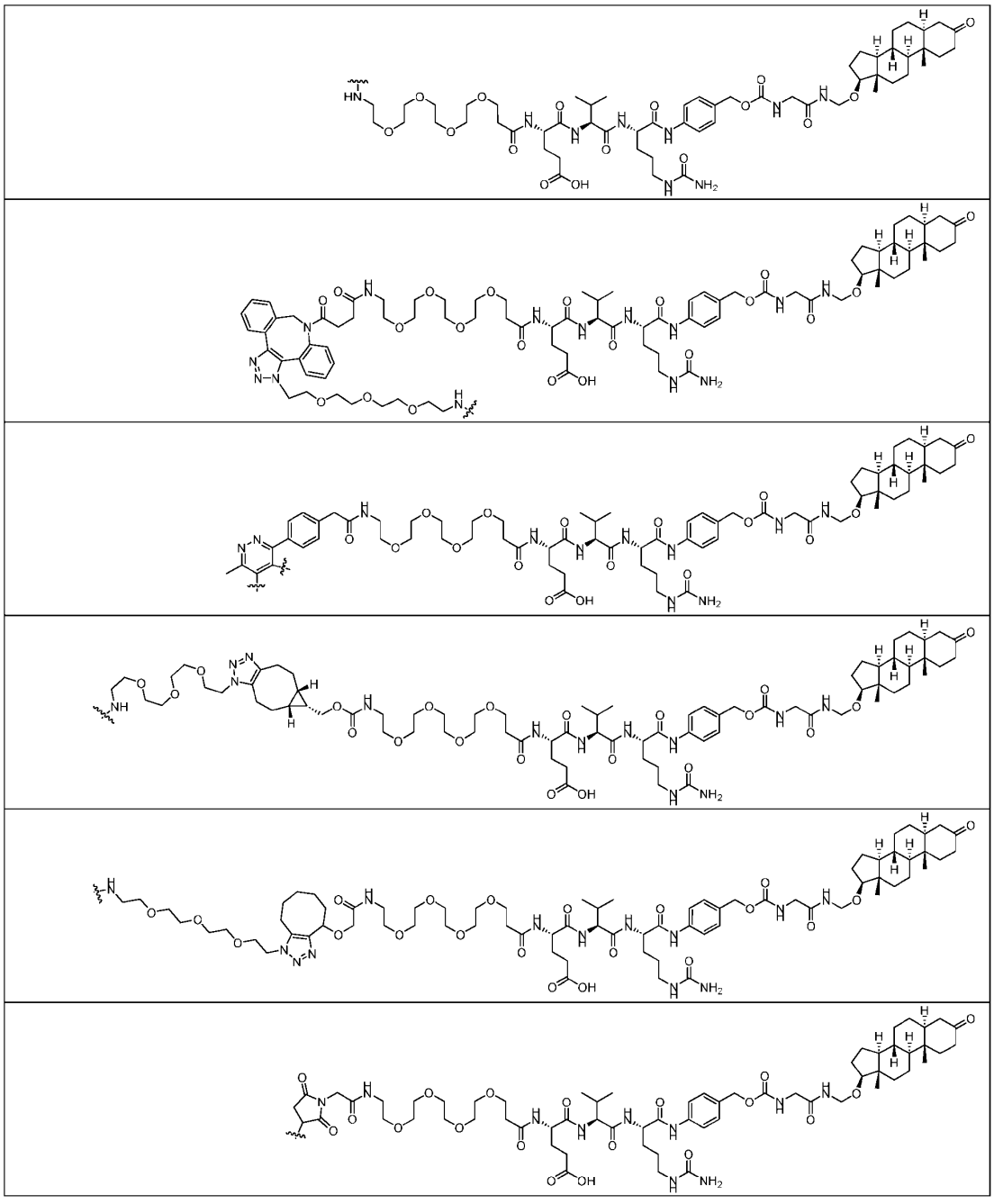
[0239] In some embodiments, the SP2 is absent or selected from the group consisting of

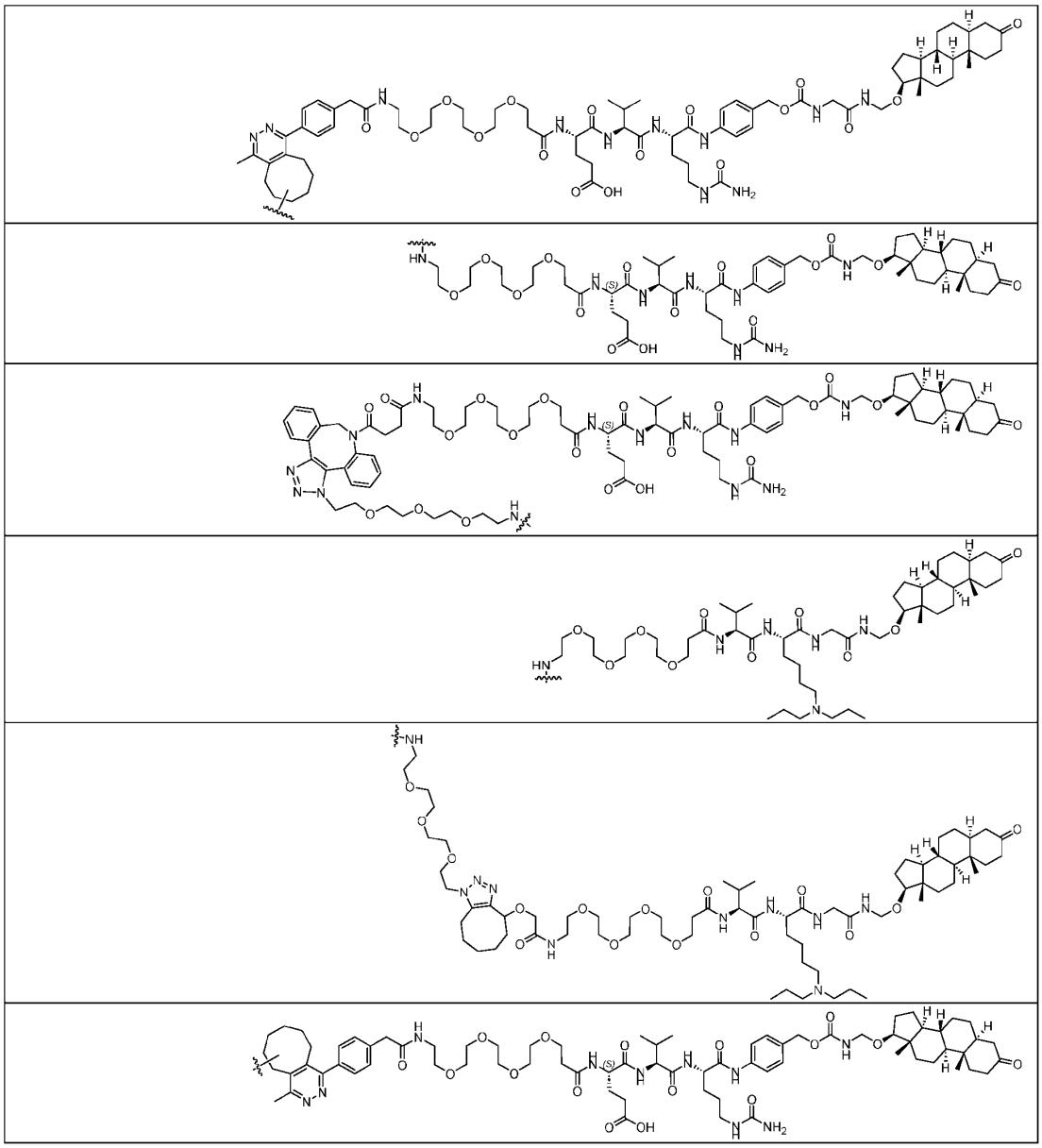


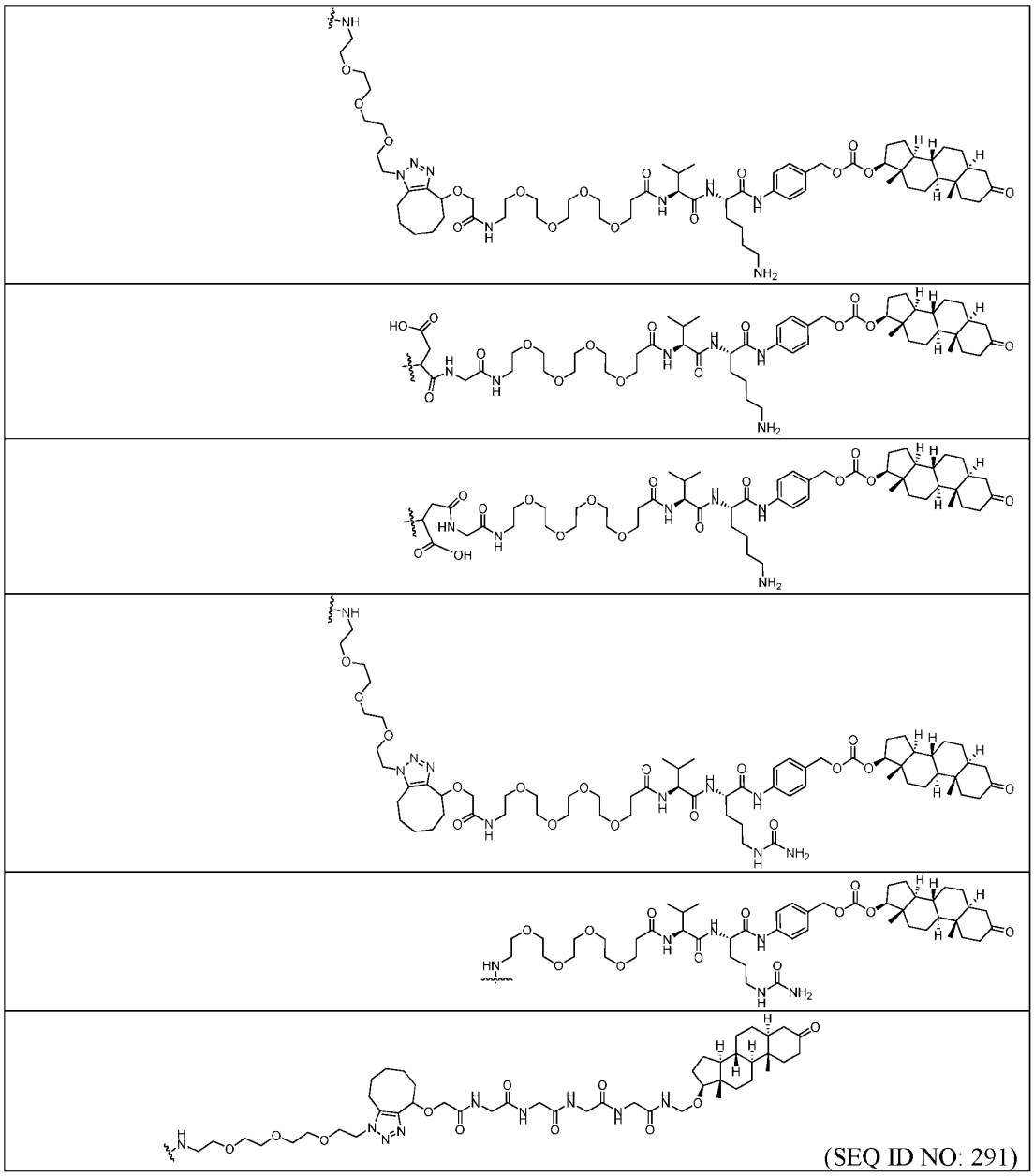
, and combinations thereof.

[0240] In some embodiments, the linker-therapeutic agent (L-P) has a structure selected from the group consisting of:

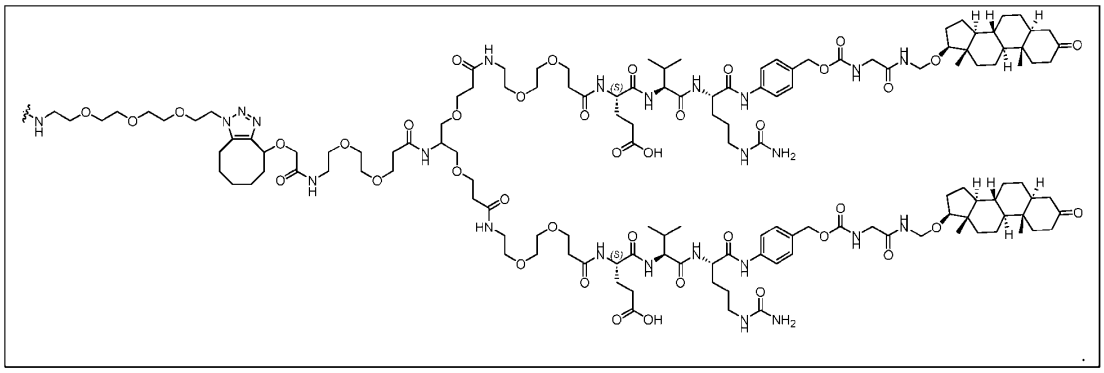








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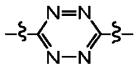
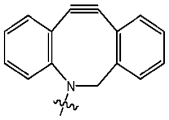
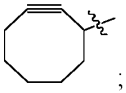


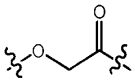
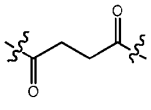
[0241] In another aspect, the present disclosure provides a compound according to Formula (L2-P) or (L2'-P):

B"-SP1-AA-SP2-P (L2-P),

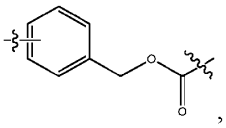
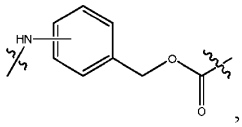
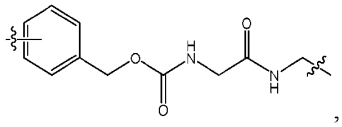
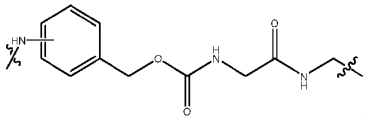
H_2N-SP1-AA-SP2-P)$_p$ (L2'-P),

or a pharmaceutically acceptable salt thereof, wherein:

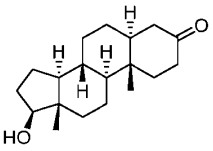
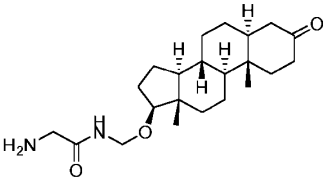
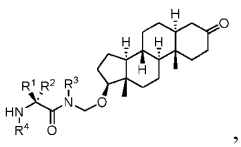
B" is selected from the group consisting of -N_3,  , ,  and  ;

SP1 is absent or a first spacer unit selected from the group consisting of  and  ; a $C_{1\text{-}6}$ alkyl, -$(CH_2$-CH_2-$O)_v$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O), -O-CH_2-C(O)-NH, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-NH, -NH-, -C(O)-, -NH-C(O)-, -NH-$(CH_2)_u$-, -NH-$(CH_2)_u$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-, -NH-$(CH_2$-CH_2-$O)_v$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-, -$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-C(O)-NH-, or combinations thereof; wherein subscripts u and v are independently an integer from 1 to 8;

AA is absent or a peptide unit comprising from 2 to 4 amino acids;

SP2 is absent or a second spacer unit selected from the group consisting of  ,  ,  ,  , and combinations thereof.

and

P is a therapeutic agent selected from the group consisting of DHT having the structure  and proDHT having the structure selected from the group consisting of  and  ,

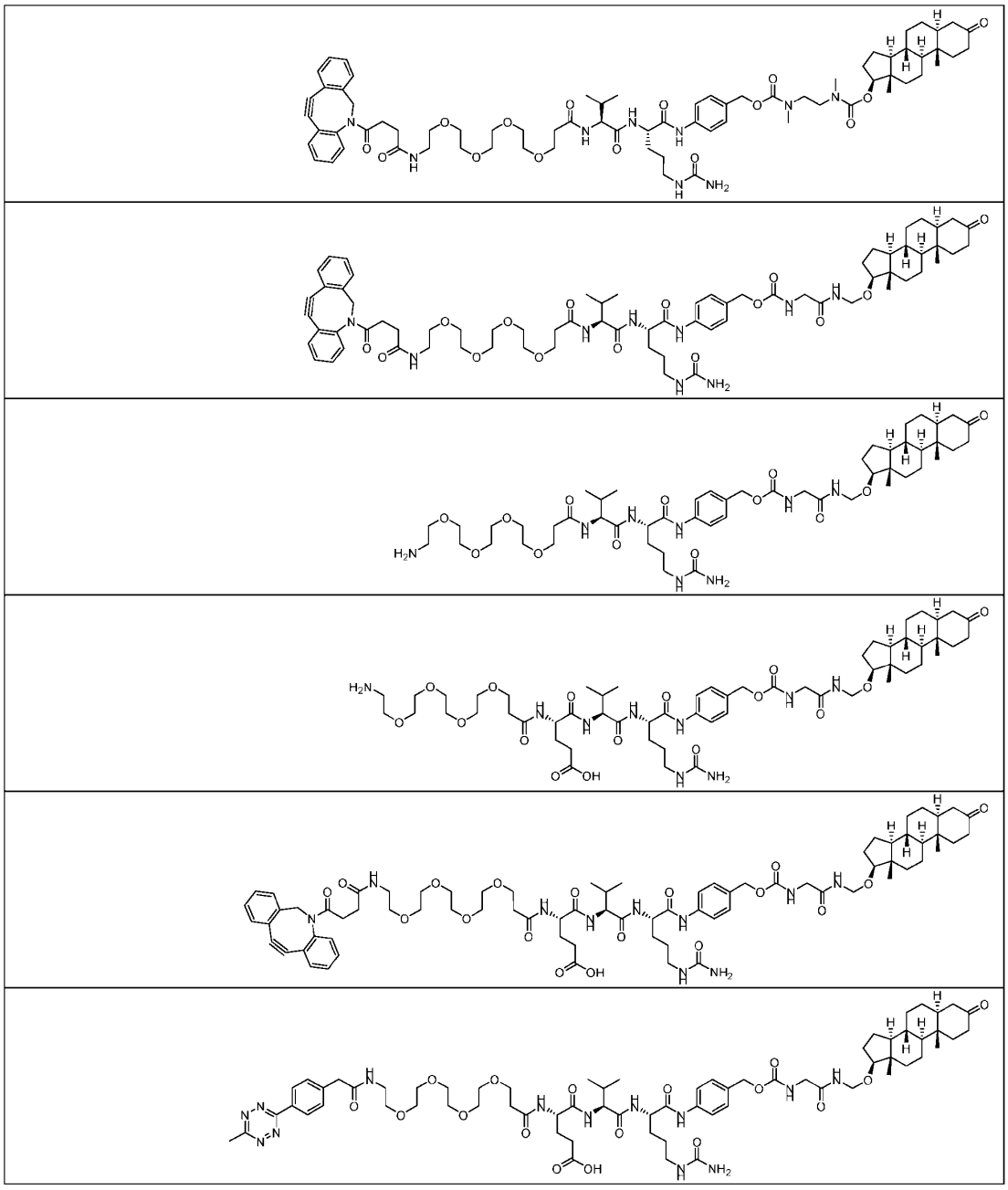
wherein R_1 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

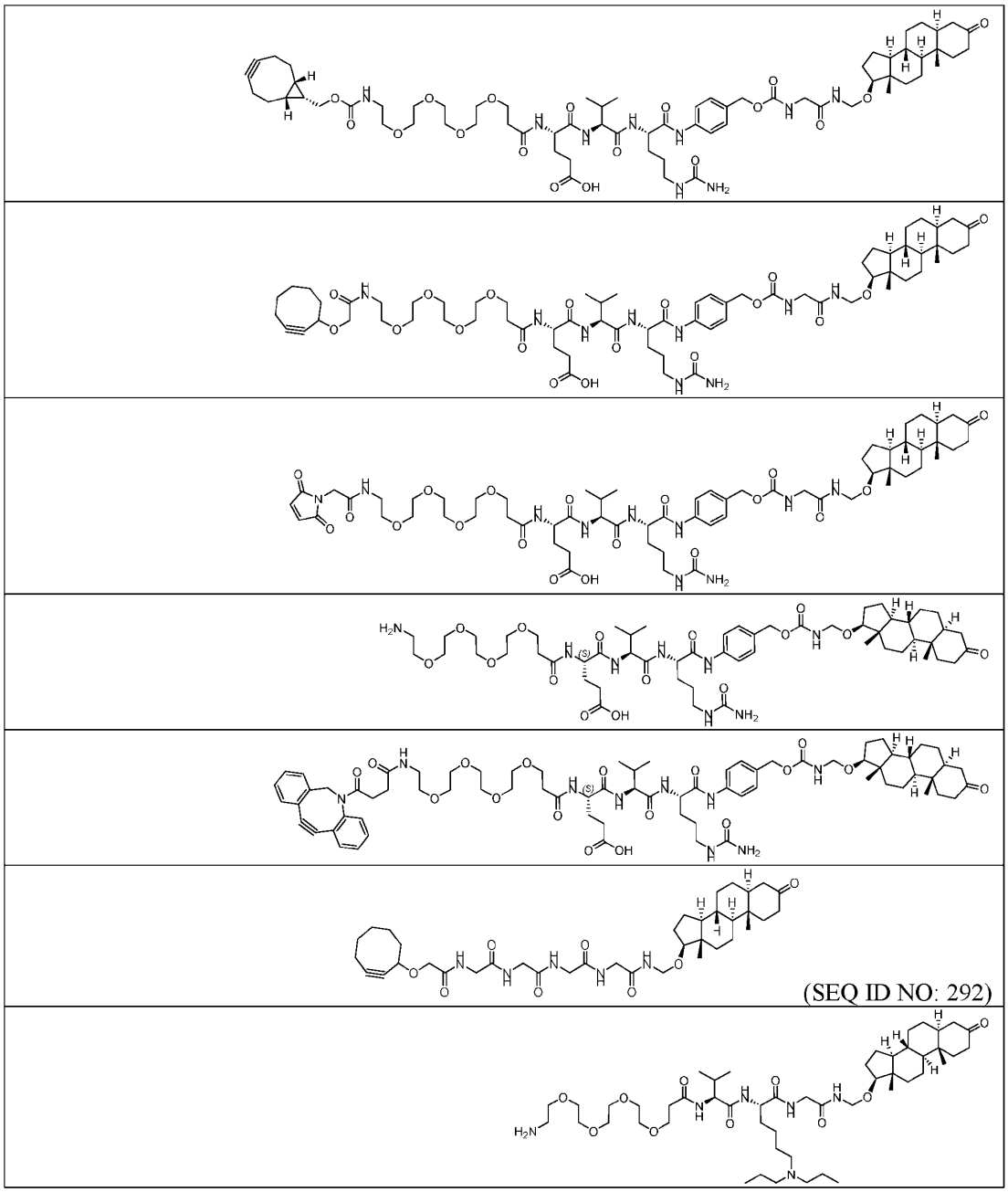
R_2 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

R_3 is selected from the group consisting of H and a C_{1-3} alkyl, and

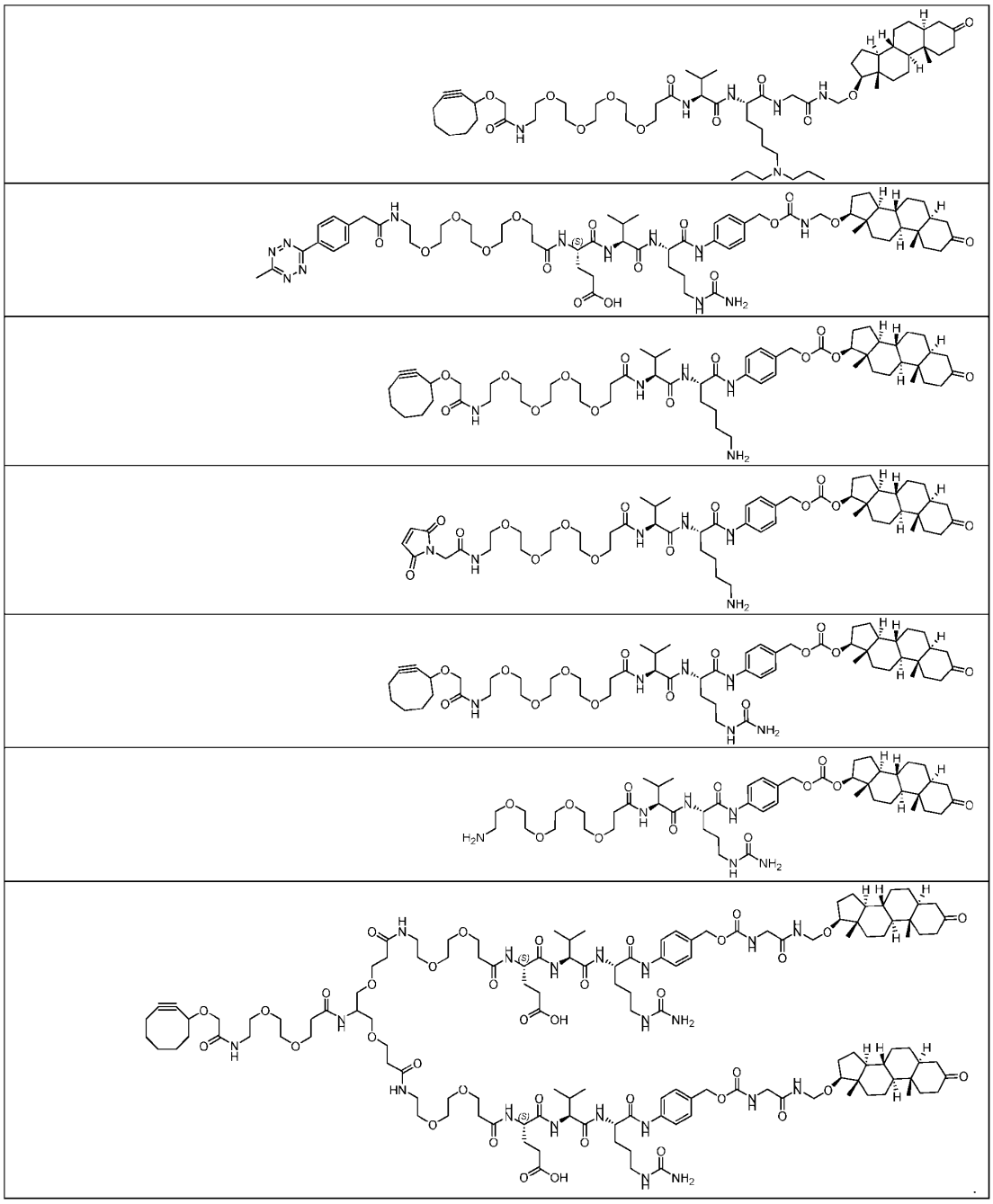
R_4 is selected from the group consisting of H and a C_{1-3} alkyl.

[0242] In one embodiment, the compound according to Formula (L2-P) or (L2'-P) has a structure selected from the group consisting of:

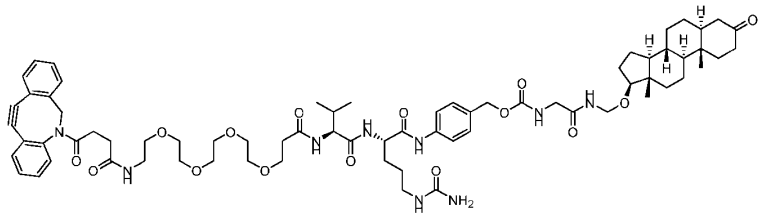




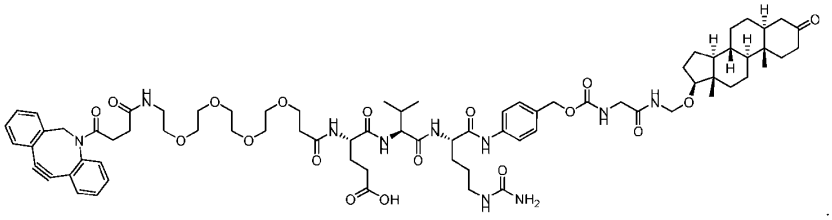
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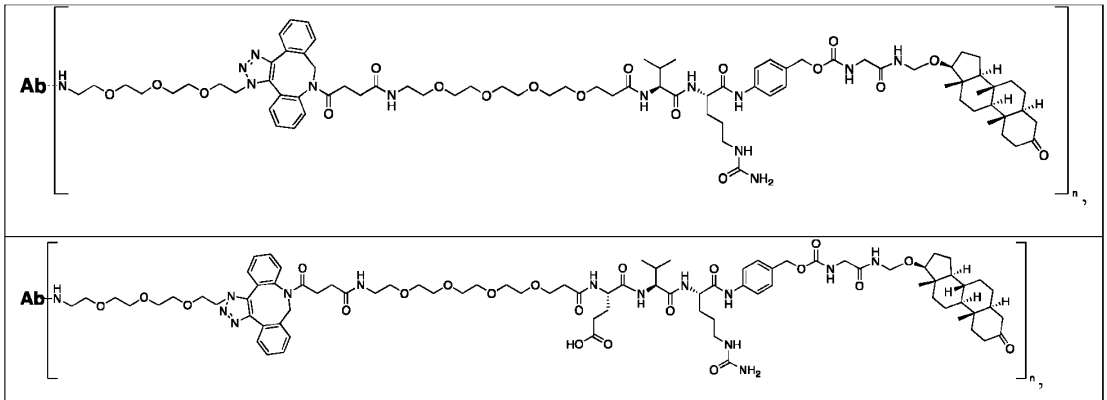
[0243] In one particular embodiment, the present disclosure provides an antibody-drug conjugate or a pharmaceutically acceptable salt thereof comprising the antigen-binding protein as described above conjugated to the linker-payload selected from the group consisting of



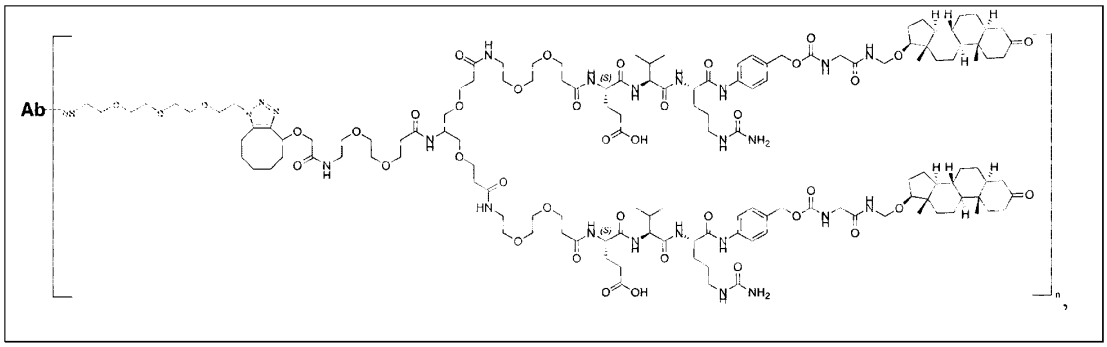
and



[0244] In one aspect, the present disclosure provides an antibody-drug conjugate having a structure selected from the group consisting of:



and

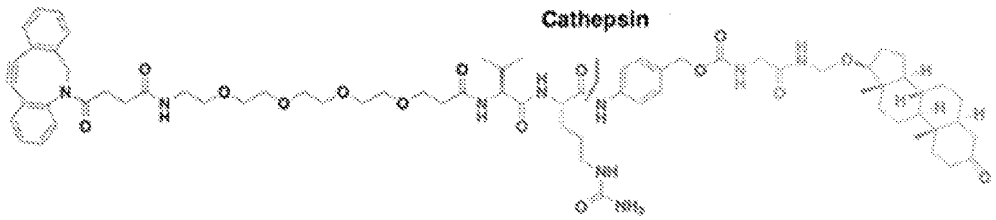


wherein Ab is the anti-hCAGNG1 antigen-binding protein as described above and n is an integer from 1 to 4.

[0245] Preparation of ADCs

[0246] The antibody drug conjugates described herein can be prepared using conjugation conditions known to those of ordinary skill in the art, (see, *e.g.*, Doronina *et al. Nature Biotechnology* 2003, 21, 7, 778, which is incorporated herein by reference in its entirety). In some embodiments an anti-hCACNG1 antigen-binding protein drug conjugate is prepared by contacting an anti-hCACNG1 antigen-binding protein AS described herein with a compound comprising the desired linker and therapeutic agent, wherein said linker possesses a moiety that is reactive with the antibody or antigen-binding protein, *e.g.*, at the desired residue of the antibody or antigen-binding protein.

[0247] In some embodiments, provided herein are processes for preparing an antibody-drug conjugate comprising contacting an anti-hCACNG1 antigen-binding protein described herein, including an azido-functionalized anti-hCACNG1 antigen binding protein, with a compound having the following formula A^1:



A1

and a transglutaminase, see, e.g., U.S. Patent No. 9,676,871, which is incorporated herein in its entirety by reference. Shown in A^1 is a cathepsin cleavage site.

[0248] In some embodiments, the protein-drug conjugates of the present disclosure are produced according to a two-step process, where Step 1 is transglutaminase-mediated site specific conjugation and Step 2 is a therapeutic agent (payload) conjugation reaction (e.g., a 1,3-cycloaddition reaction).

Step 1: Transglutaminase Mediated Site Specific Conjugation

[0249] In some embodiments, anti-hCACNG1 antigen-binding proteins of the disclosure may be modified in accordance with known methods to provide glutaminyl modified proteins. Techniques for conjugating antibodies and primary amine compounds are known in the art. Site specific conjugation techniques are employed herein to direct conjugation to glutamine using glutamine conjugation via transglutaminase (*see e.g.*, Schibli, *Angew Chemie* Inter Ed. 2010, 49, 9995).

[0250] Primary amine-comprising compounds (e.g., linkers L1) of the present disclosure can be conjugated to one or more glutamine residues of an antigen-binding protein (e.g., an anti-hCACNG1 antigen-binding protein) via transglutaminase-based chemo-enzymatic conjugation (*see, e.g.,* Dennler et al., *Protein Conjugate Chem.* 2014, 25, 569-578, and WO 2017/147542). For example, in the presence of transglutaminase, one or more glutamine residues of an antibody can be coupled to a primary amine linker compound. Briefly, in some embodiments, an anti-hCACNG1 antigen-binding protein according to the disclosure having a glutamine residue (*e.g.*, a Gln 295, i.e. Q295 residue) is treated with a primary amine-containing linker L1, described above, in the presence of the enzyme transglutaminase. In certain embodiments, the antigen-binding protein is aglycosylated. In certain embodiments, the antigen-binding protein is deglycosylated.

[0251] In certain embodiments, the antigen-binding protein comprises at least one glutamine residue in at least one polypeptide chain sequence. In certain embodiments, the binding agent comprises two heavy chain polypeptides, each with one Gln 295 residue. In further embodiments, the binding agent comprises one or more glutamine residues at a site other than a heavy chain 295.

[0252] In some embodiments, a binding agent, such as an antibody, can be prepared by site-directed mutagenesis to insert a glutamine residue at a site without resulting in disabled antibody function or binding. For example, included herein are antibodies bearing Asn297Gln (N297Q)

mutation(s) as described herein. In some embodiments, an antibody having a Gln 295 residue and/or an N297Q mutation contains one or more additional naturally occurring glutamine residues in their variable regions, which can be accessible to transglutaminase and therefore capable of conjugation to a linker or a linker-payload. An exemplary naturally occurring glutamine residue can be found, *e.g.*, at Q55 of the light chain. In such instances, the binding agent, e.g., antibody, conjugated via transglutaminase can have a higher than expected LAR value (*e.g.*, a LAR higher than 4). Any such antibodies can be isolated from natural or artificial sources.

[0253] In certain embodiments of the disclosure, the linker-antibody ratio or LAR is from 1, 2, 3, 4, 5, 6, 7, or 8 linker L1 molecules per antibody. In some embodiments, the LAR is from 1 to 8. In some embodiments, the LAR is from 1 to 6. In certain embodiments, the LAR is from 2 to 4. In some cases, the LAR is from 2 to 3. In certain cases, the LAR is from 0.5 to 3.5. In some embodiments, the LAR is about 1, or about 1.5, or about 2, or about 2.5, or about 3, or about 3.5. In some embodiments, the LAR is 2. In some embodiments, the LAR is 4.

Step 2: Payload Conjugation Reaction

[0254] In certain embodiments, linkers L1 according to the present disclosure comprise at least one reactive group B' capable of further reaction after transglutamination. In these embodiments, the glutaminyl-modified antigen-binding protein is capable of further reaction with a reactive payload compound or a reactive linker-payload compound (e.g., L2-P as disclosed herein), to form an antigen-binding protein-payload conjugate. More specifically, the reactive linker-payload compound L2-P may comprise a reactive group B" that is capable of reacting with the reactive group B' of the linker L1. In certain embodiments, a reactive group B' according to the present disclosure comprises a moiety that is capable of undergoing a 1,3-cycloaddition reaction. In certain embodiments, the reactive group B' is an azide. In certain embodiments, the reactive group B" comprises an alkyne (e.g., a terminal alkyne, or an internal strained alkyne). In certain embodiments of the present disclosure the reactive group B' is compatible with the antigen-binding protein and transglutamination reaction conditions.

[0255] In certain embodiments of the disclosure, linker L1 molecules comprise one reactive group B'. In certain embodiments of the disclosure, linker L1 molecules comprise more than one reactive group B'.

[0256] In certain embodiments, the reactive linker-payload L2-P comprises one payload molecule (n = 1). In certain other embodiments, the reactive linker-payload L2-P comprises two or more payload molecules (n ≥ 2). In certain embodiments, the reactive linker-payload L2-P comprises from 1 to 12 payload molecules, or from 1 to 10 payload molecules, or from 1 to 8 payload molecules, or from 1 to 6 payload molecules, or from 1 to 4 payload molecules, or from 1 to 2 payload molecules.

[0257] In certain embodiments, the reactive linker-payload L2-P comprises one payload molecule. When such L2-P is reacted with an Ab-L1-B', the DAR will be about equal to the LAR of the Ab-L1-B'. For example, if L2-P comprising one payload molecule is reacted with an Ab-L1-B' having a LAR of 4 (e.g., via Q295 and N297Q transglutamination), the resulting protein-drug conjugate will have a DAR of 4.

[0258] In certain embodiments, the reactive linker-payload L2-P comprises 2 payload molecules. When such L2-P is reacted with an Ab-L1-B', the DAR will be about 2 times the LAR of the Ab-L1-B'. For example, if L2-P comprising 2 payload molecules is reacted with an Ab-L1-B' having a LAR of 4 (e.g., via Q295 and N297Q transglutamination), the resulting protein-drug conjugate will have a DAR of 8.

[0259] In certain embodiments of the disclosure, the drug-antibody ratio or DAR (e.g., abbreviated as the lower case letter n) is from about 1 to about 24, or from about 1 to about 20, or from about 1 to about 16, or from about 1 to about 12, or from about 1 to about 10, or from about 1 to about 8, or about 1, 2, 3, 4, 5, 6, 7, or 8 payload molecules per antibody. In some embodiments, the DAR is from 1 to 24. In some embodiments, the DAR is from 1 to 16. In some embodiments, the DAR is from 1 to 8. In some embodiments, the DAR is from 1 to 6. In certain embodiments, the DAR is from 2 to 4. In some cases, the DAR is from 2 to 3. In certain cases, the DAR is from 0.5 to 3.5. In certain cases, the DAR is from 10 to 14. In certain cases, the DAR is from 14 to 18. In certain cases, the DAR is from 20 to 24.5. In some embodiments, the DAR is about 1, or about 1.5, or about 2, or about 2.5, or about 3, or about 3.5. In some embodiments, the DAR is 2. In some embodiments, the DAR is 4. In some embodiments, the DAR is 8. In some embodiments, the DAR is 12. In some embodiments, the DAR is 16. In some embodiments, the DAR is 24.

[0260] In one aspect, the present disclosure provides a method of producing a compound having a structure according to Formula (A):

$$Ab\text{-}(L1\text{-}B\text{-}L2\text{-}P)_n \quad (A),$$

or a pharmaceutically acceptable salt thereof, wherein:

Ab is an anti-hCACNG1 antigen-binding protein described herein;

L1 is a first linker covalently bound to the side chain of a glutamine residue of the Ab;

B is a moiety comprising a triazole;

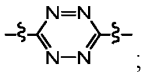
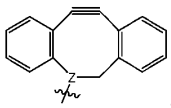
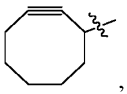
L2 is a second linker covalently bound to the therapeutic agent P;

P is a therapeutic agent selected from the group consisting of DHT and proDHT, and

n is an integer from 1 to 8, wherein the method comprises the steps of:

a) contacting, in the presence of a transglutaminase, the A comprising at least one glutamine residue with at least one compound L1-B';

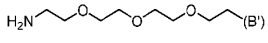
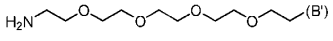
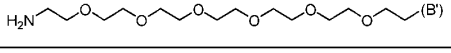
b) contacting the product of step a) with one or more equivalents of a compound B"-L2-P, wherein the group B" is capable of covalently attaching to the group B',

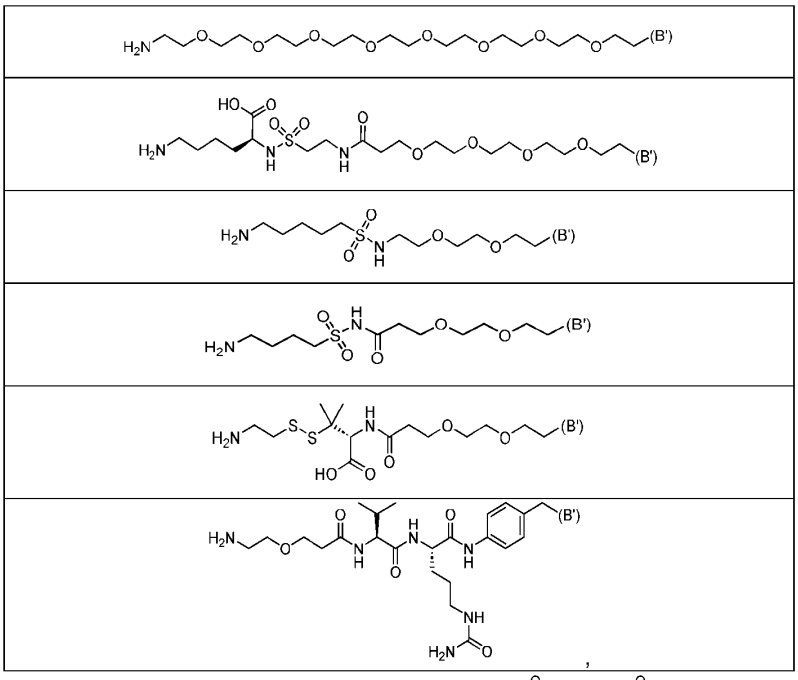
wherein one of the groups B' and B" is selected from $-N_3$ and  ; and the other of the groups B' and B" is selected from ,  ; and  , where Z is C or N; and

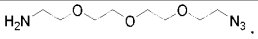
c) isolating the produced compound of Formula (A).

[0261] In one embodiment, the A has glutamine residues at positions 295 (Q295) and 297 (N297Q).

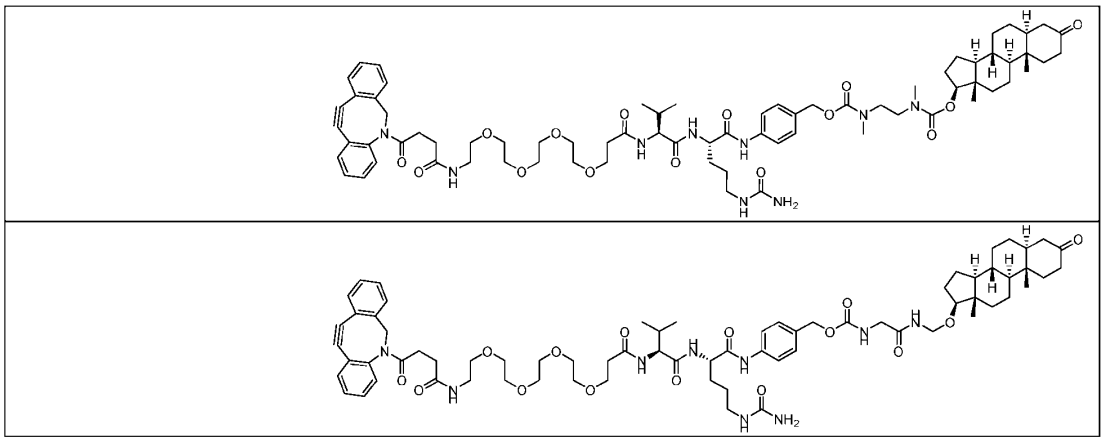
[0262] In one embodiment, the L1-B' has the structure selected from the group consisting of:

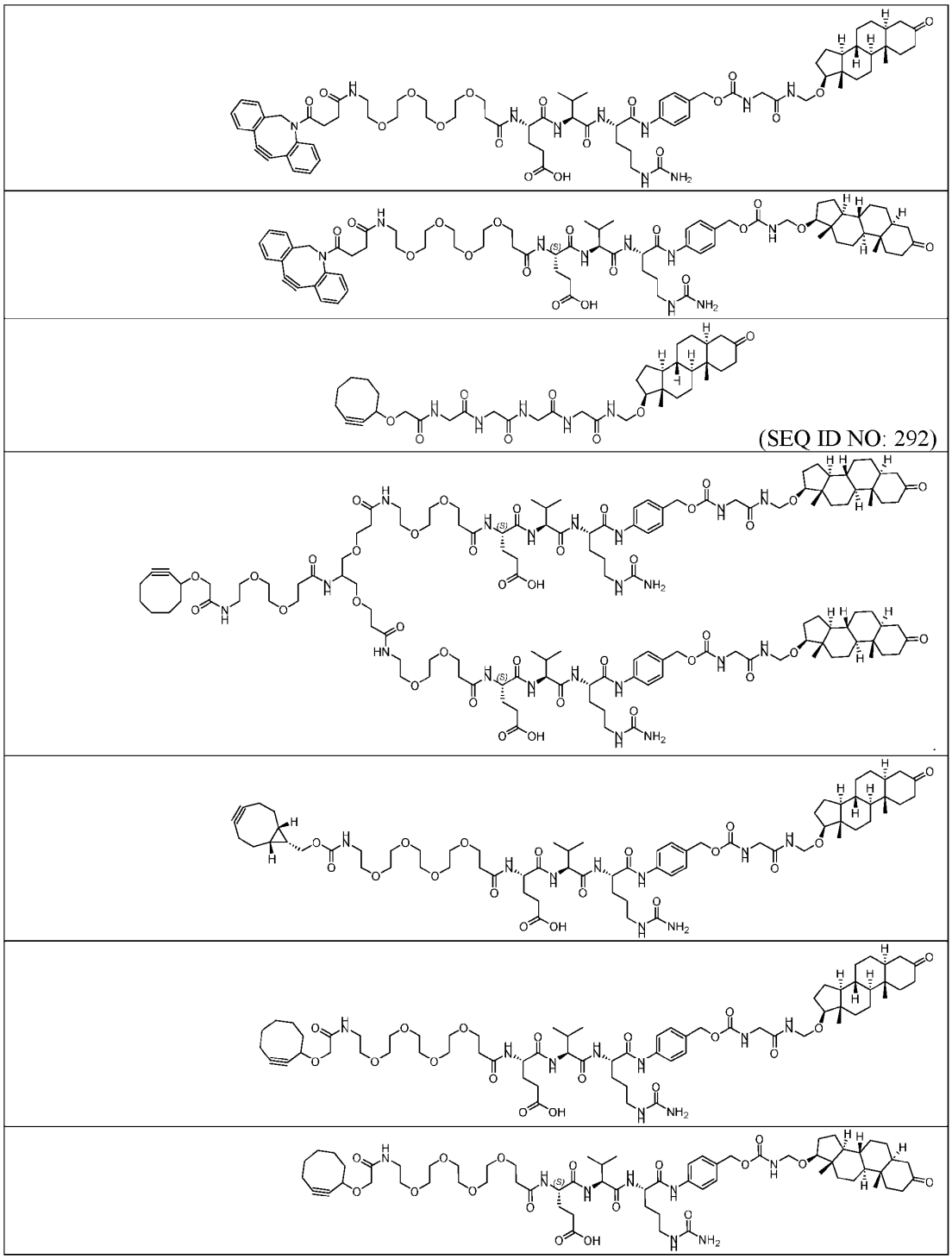






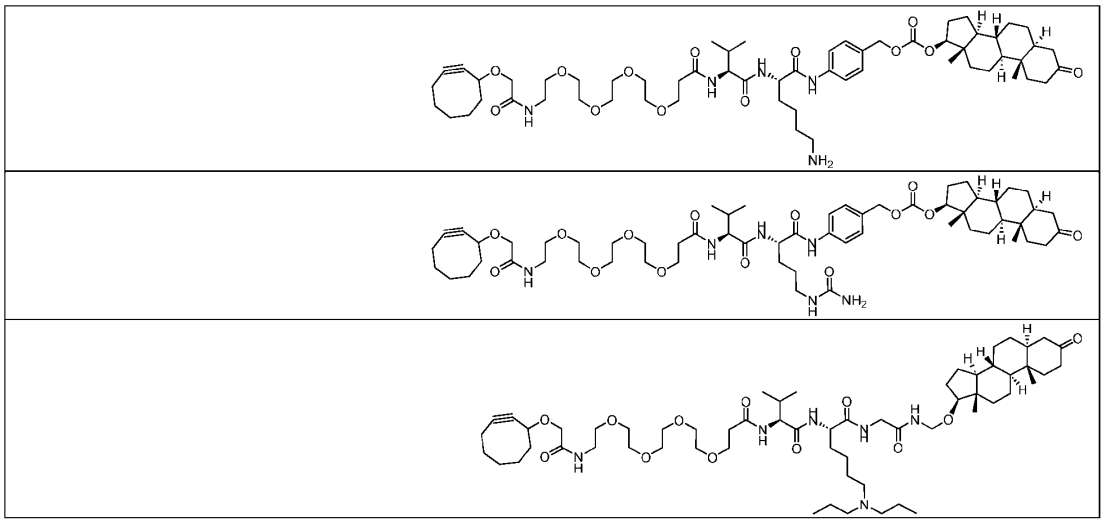
[0263] In one embodiment, the L1-B' has the structure  .

[0264] In one embodiment, the compound B"-L2-P has the structure selected from the group consisting of:

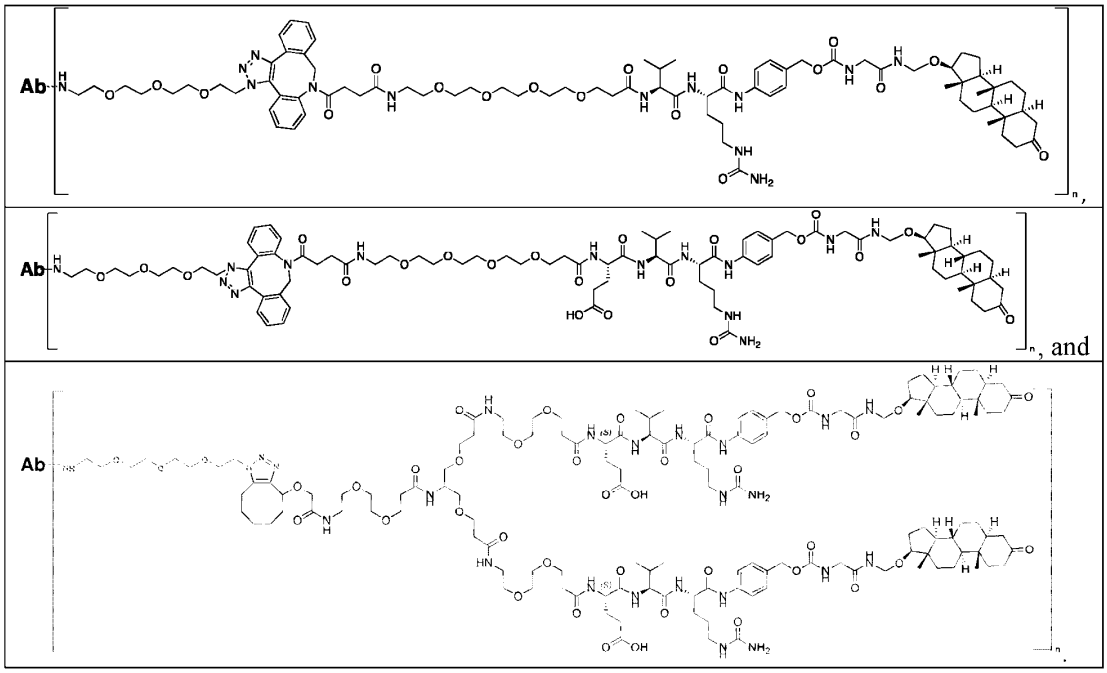




(SEQ ID NO: 292)



[0265] In one embodiment, the compound of Formula (A) has the structure selected from the group consisting of:



[0266] In another aspect, the present disclosure provides a method of producing antigen-binding protein-drug conjugates in a single step. In such a process, the antigen-binding protein according to

the disclosure (i.e., an anti-hCACNG1 antigen-binding protein described herein) is treated with a primary amine-containing linker-payload L-P, described above, in the presence of the enzyme transglutaminase. In certain embodiments, the antigen-binding protein is aglycosylated. In certain embodiments, the antigen-binding protein is deglycosylated.

[0267] In one aspect, the present disclosure provides a method of producing a compound having a structure according to Formula (I):

$A - [L - P]_y$ (I),

wherein A is the antigen-binding protein;

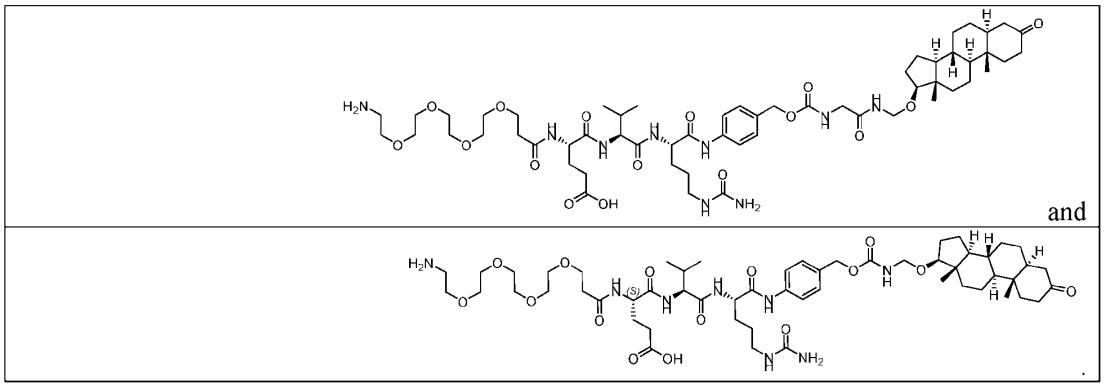
L is a linker;

P is a therapeutic agent selected from the group consisting of DHT and proDHT, and

y is an integer from 1 to 8, wherein the method comprises the steps of:

c) contacting, in the presence of a transglutaminase, the A comprising at least one glutamine residue with at least one compound L-P, wherein the compound L-P has at least one terminal amine moiety, and
d) isolating the produced compound of Formula (I).

[0268] In one embodiment, the compound L-P has the structure selected from the group consisting of:



Therapeutic Formulation and Administration

[0269] Also described herein are pharmaceutical compositions comprising the antigen-binding molecules as described herein. In some embodiments, pharmaceutical compositions may be formulated with suitable carriers, excipients, and other agents that provide improved transfer, delivery, tolerance, and the like. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as LIPOFECTIN™, Life Technologies, Carlsbad, CA), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. See also Powell et al. "Compendium of excipients for parenteral formulations" PDA (1998) J Pharm Sci Technol 52:238-311.

[0270] The dose of antigen-binding molecule administered to a patient may vary depending upon the age and the size of the patient, target disease, conditions, route of administration, and the like. The preferred dose is typically calculated according to body weight or body surface area. When an antigen-binding molecule as described herein is used for therapeutic purposes in an adult patient, it may be advantageous to intravenously administer the antigen-binding molecule as described herein normally at a single dose of about 0.01 to about 20 mg/kg body weight, more preferably about 0.02 to about 7, about 0.03 to about 5, or about 0.05 to about 3 mg/kg body weight. Depending on the severity of the condition, the frequency and the duration of the treatment can be adjusted. Effective dosages and schedules for administering a bispecific antigen-binding molecule may be determined empirically; for example, patient progress can be monitored by periodic assessment, and the dose adjusted accordingly. Moreover, interspecies scaling of dosages can be performed using well-known methods in the art (*e.g.*, Mordenti *et al.*, 1991, *Pharmaceut. Res. 8*:1351).

[0271] Various delivery systems are known and can be used to administer the pharmaceutical composition as described herein, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the mutant viruses, receptor mediated endocytosis (see, e.g., Wu et al., 1987, J. Biol. Chem. 262:4429-4432). Methods of introduction include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The composition may be administered by any convenient route, for

example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

[0272] A pharmaceutical composition as described herein can be delivered subcutaneously or intravenously with a standard needle and syringe. In addition, with respect to subcutaneous delivery, a pen delivery device readily has applications in delivering a pharmaceutical composition as described herein. Such a pen delivery device can be reusable or disposable. A reusable pen delivery device generally utilizes a replaceable cartridge that contains a pharmaceutical composition. Once all of the pharmaceutical composition within the cartridge has been administered and the cartridge is empty, the empty cartridge can readily be discarded and replaced with a new cartridge that contains the pharmaceutical composition. The pen delivery device can then be reused. In a disposable pen delivery device, there is no replaceable cartridge. Rather, the disposable pen delivery device comes prefilled with the pharmaceutical composition held in a reservoir within the device. Once the reservoir is emptied of the pharmaceutical composition, the entire device is discarded.

[0273] Numerous reusable pen and autoinjector delivery devices have applications in the subcutaneous delivery of a pharmaceutical composition as described herein. Examples include, but are not limited to AUTOPEN™ (Owen Mumford, Inc., Woodstock, UK), DISETRONIC™ pen (Disetronic Medical Systems, Bergdorf, Switzerland), HUMALOG MIX 75/25™ pen, HUMALOG™ pen, HUMALIN 70/30™ pen (Eli Lilly and Co., Indianapolis, IN), NOVOPEN™ I, II and III (Novo Nordisk, Copenhagen, Denmark), NOVOPEN JUNIOR™ (Novo Nordisk, Copenhagen, Denmark), BD™ pen (Becton Dickinson, Franklin Lakes, NJ), OPTIPEN™, OPTIPEN PRO™, OPTIPEN STARLET™, and OPTICLIK™ (sanofi-aventis, Frankfurt, Germany), to name only a few. Examples of disposable pen delivery devices having applications in subcutaneous delivery of a pharmaceutical composition as described herein include, but are not limited to the SOLOSTAR™ pen (sanofi-aventis), the FLEXPEN™ (Novo Nordisk), and the KWIKPEN™ (Eli Lilly), the SURECLICK™ Autoinjector (Amgen, Thousand Oaks, CA), the PENLET™ (Haselmeier, Stuttgart, Germany), the EPIPEN (Dey, L.P.), and the HUMIRA™ Pen (Abbott Labs, Abbott Park IL), to name only a few.

[0274] In certain situations, the pharmaceutical composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201). In another embodiment, polymeric materials can be used; see, Medical Applications of Controlled Release, Langer and Wise (eds.), 1974, CRC Pres., Boca Raton, Florida. In yet another embodiment, a controlled release system can be placed in proximity of the composition's target, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, 1984, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138). Other controlled release systems are discussed in the review by Langer, 1990, Science 249:1527-1533.

[0275] The injectable preparations may include dosage forms for intravenous, subcutaneous, intracutaneous and intramuscular injections, drip infusions, etc. These injectable preparations may be prepared by methods publicly known. For example, the injectable preparations may be prepared, *e.g.*, by dissolving, suspending or emulsifying the antibody or its salt described above in a sterile aqueous medium or an oily medium conventionally used for injections. As the aqueous medium for injections, there are, for example, physiological saline, an isotonic solution containing glucose and other auxiliary agents, etc., which may be used in combination with an appropriate solubilizing agent such as an alcohol (e.g., ethanol), a polyalcohol (e.g., propylene glycol, polyethylene glycol), a nonionic surfactant [e.g., polysorbate 80, HCO-50 (polyoxyethylene (50 mol) adduct of hydrogenated castor oil)], etc. As the oily medium, there are employed, e.g., sesame oil, soybean oil, etc., which may be used in combination with a solubilizing agent such as benzyl benzoate, benzyl alcohol, etc. The injection thus prepared is preferably filled in an appropriate ampoule.

[0276] Advantageously, the pharmaceutical compositions for oral or parenteral use described above are prepared into dosage forms in a unit dose suited to fit a dose of the active ingredients. Such dosage forms in a unit dose include, for example, tablets, pills, capsules, injections (ampoules), suppositories, etc. The amount of the aforesaid antibody contained is generally about 5 to about 500 mg per dosage form in a unit dose; especially in the form of injection, it is preferred that the aforesaid antibody is contained in about 5 to about 100 mg and in about 10 to about 250 mg for the other dosage forms.

Therapeutic and Diagnostic Uses thereof

[0277] Disclosed herein are also methods comprising administering to a subject in need thereof a therapeutic composition comprising an anti-hCACNG1 antibody, antigen-binding fragment thereof or an antibody-drug conjugate comprising an anti-hCACNG1 antibody (e.g., an anti-hCACNG1 antibody, or ADC comprising any of the HCVR/LCVR or CDR sequences as set forth in Table 1 herein). The therapeutic composition can comprise any of the anti-hCACNG1 antibodies, antigen-binding fragments thereof, or ADCs disclosed herein, and a pharmaceutically acceptable carrier or diluent.

[0278] The antibodies, antigen-binding fragment thereof, or an antibody-drug conjugate comprising an anti-hCACNG1 antibody as described herein may be useful, *inter alia*, for the treatment, prevention and/or amelioration of any disease or disorder associated with skeletal muscle tissue. For example, the antibodies and ADCs as described herein may be useful for the treatment of muscle wasting disorders (e.g., cachexia, glucocorticoid-induced muscle loss, heart failure induced muscle loss, HIV wasting, disuse, aging, etc.) and/or muscular dystrophies/myopathies.

[0279] The anti-hCACNG1 antibodies as described herein have various utilities. For example, in some embodiments, anti-hCACNG1 antibodies as described herein may be used in diagnostic assays for CACNG1, e.g., detecting its expression in specific cells, tissues, etc., e.g., as a reagent to identify/label skeletal muscle fibers. Various diagnostic and prognostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases (Zola (1987) Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. pp. 147-1581). The antibodies used in the assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. Any method known in the art for conjugating the antibody to the detectable moiety may be employed.

[0280] In another embodiment, provided is a method of treatment of a disease, such as a muscle wasting disorder. The method may include the step of providing an antibody or CACNG1 antigen-binding fragment thereof, as described above, to a subject requiring said treatment.

Example 1: Exemplary CACNG1 antibodies

[0281] *Generation of anti-human CACNG1 antibodies*

[0282] Anti-human CACNG1 antibodies were obtained by immunizing a mouse (e.g., an engineered mouse comprising DNA encoding human immunoglobulin heavy and human kappa light chain variable regions), with human CACNG1.

[0283] Following immunization, splenocytes were harvested from each mouse and either (1) fused with mouse myeloma cells to preserve their viability and form hybridoma cells and screened for human CACNG1 specificity, or (2) B-cell sorted (as described in US 2007/0280945A1) using a either a human CACNG1 fragment as the sorting reagent that binds and identifies reactive antibodies (antigen-positive B cells).

[0284] Chimeric antibodies to human CACNG1 were initially isolated having a human variable region and a mouse constant region using, *e.g.*, VELOCIMMUNE technology as described in US Patent No. 7,105,348; US Patent No. 8,642,835; and US 9,622,459, each of which is incorporated herein by reference.

[0285] In some antibodies, for testing purposes, mouse constant regions were replaced with a desired human constant region, for example wild-type human CH or modified human CH (*e.g.* IgG1, IgG2 or IgG4 isotypes), and light chain constant region (CL), to generate a fully human anti-hCACNG1 antibody, or antigen binding portion thereof. While the constant region selected may vary according to specific use, high affinity antigen-binding and target specificity characteristics reside in the variable region.

[0286] Certain biological properties of the exemplary anti-human CACNG1 antibodies generated in accordance with the methods of this Example are described in detail in the Examples set forth below.

[0287] *Heavy and Light Chain Variable Region Amino Acid and Nucleic Acid Sequences of anti-hCACNG1 antibodies*

[0288] Table 1 sets forth sequence identifiers or sequences of a nucleic acid (NA) sequence encoding, and in parentheses an amino acid (AA) sequence of, a heavy or light chain variable region (HCVR or LCVR, respectively), or a heavy or light chain CDR (HCDR and LCDR,

respectively) of selected anti-hCACNG1 antibodies used to generate the therapeutic anti-hCACNG1 proteins disclosed herein.

Table 1: anti-hCACNG1 Sequence Identifiers or Sequences

	SEQ ID NOs:							
Antibody Designation	HCVR NA (AA)	HCDR1 NA (AA)	HCDR2 NA (AA)	HCDR3 NA (AA)	LCVR NA (AA)	LCDR1 NA (AA)	LCDR2 NA (AA)	LCDR3 NA (AA)
1 anti-hCACNG1 Ab 18/ anti-hCACNG1 Ab 5/ anti-hCACNG1 Ab 2	1 (2)	3 (4)	5 (6)	7 (8)	9 (10)	11 (12)	GCTGCATCC (AAS)	15 (16)
2 anti-hCACNG1 Ab 6/ anti-hCACNG1 Ab 14	17 (18)	19 (20)	21 (22)	23 (24)	25 (26)	27 (28)	GCTACATCC (ATS)	31 (32)
3 anti-hCACNG1 Ab 8/ anti-hCACNG1 Ab 14	33 (34)	35 (36)	37 (38)	39 (40)	41 (42)	43 (44)	AAGGCGTCT (KAS)	47 (48)
4 anti-hCACNG1 Ab 7/ anti-hCACNG1 Ab 14	49 (50)	51 (52)	53 (54)	55 (56)	57 (58)	59 (60)	GGTGCATCC (GAS)	63 (64)
5 anti-hCACNG1 Ab 10/ anti-hCACNG1 Ab 17	65 (66)	67 (68)	69 (70)	71 (72)	73 (74)	75 (76)	GCTGCATCC (AAS)	79 (80)
6	81 (82)	83 (84)	85 (86)	87 (88)	89 (90)	91 (92)	GCTGCATCC (AAS)	95 (96)

anti-hCACNG1 Ab 3								
7 anti-hCACNG1 Ab 20/ anti-hCACNG1 Ab 1	97 (98)	99 (100)	101 (102)	103 (104)	105 (106)	107 (108)	GCTGCATCC (AAS)	111 (112)
8 anti-hCACNG1 Ab 4	113 (114)	115 (116)	117 (118)	119 (120)	121 (122)	123 (124)	GGTGCATCC (GA)	127 (128)
9 anti-hCACNG1 Ab 11	129 (130)	131 (132)	133 (134)	135 (136)	137 (138)	139 (140)	GGTGCATCC (GAS)	143 (144)
10 anti-hCACNG1 Ab 12	145 (146)	147 (148)	149 (150)	151 (152)	153 (154)	155 (156)	AGGAATAAT (RN)	159 (160)
11 anti-hCACNG1 Ab 9/ anti-hCACNG1 Ab 13	161 (162)	163 (164)	165 (166)	167 (168)	169 (170)	171 (172)	GACAATAAT (DNN)	175 (176)
12 anti-hCACNG1 Ab 19	177 (178)	179 (180)	181 (182)	183 (184)	185 (186)	187 (188)	GGTGCATCC (GAS)	191 (192)
13 anti-hCACNG1 Ab 21	293 (294)	295 (36)	296 (297)	298 (299)	300 (301)	302 (303)	TATAACAGC (YNS)	304 (305)
14	313 (314)	315 (316)	317 (318)	319 (152)	321 (322)	323 (324)	AGAAATAAT (RNN)	325 (326)

anti-hCACNG1 Ab 22								

Example 2: Biacore binding kinetics of anti-CACNG1 monoclonal antibodies to human CACNG1 nanodisc in an antigen capture format at 25°C

[0289] Equilibrium dissociation constants (K_D values) for nanodisc embedded human CACNG1 expressed with a C-terminal PADRE-Flag-His tag (human CACNG1 nanodisc) binding to purified anti-hCACNG1 antibodies were determined using a real-time surface plasmon resonance biosensor technology with a Biacore T200 instrument. The CM5 Biacore sensor surface was derivatized by amine coupling with a monoclonal mouse anti-His antibody (Cytiva; Marlborough, MA). All Biacore binding studies were performed in a buffer composed of 0.01M HEPES, 0.15M NaCl, 1mM $CaCl_2$, 0.5mM $MgCl_2$, pH 7.4 (HBS-N++ running buffer). Different concentrations of anti-hCACNG1 antibodies (ranging from 300nM to 12nM in 5-fold serial dilutions) prepared in HBS-N++ running buffer were injected over the captured human CACNG1 nanodisc at a flow rate of 30µL/minute. Antibody association was monitored for 2 minutes while dissociation was monitored in HBS-N++ running buffer for 5 minutes. At the end of each cycle, the human CACNG1 nanodisc capture surface was regenerated using three 10 second injections of 10mM Gly pH1.5. All binding kinetics experiments were performed at 25°C.

[0290] The specific SPR-Biacore sensorgrams were obtained by a double referencing procedure. The double referencing was performed by first subtracting the signal of each injection over a reference surface (anti-His) from the signal over the experimental surface (anti-His captured human CACNG1 nanodisc) thereby removing contributions from refractive index changes. In addition, running buffer injections were performed to allow subtraction of the signal changes resulting from the dissociation of captured antibodies from the coupled anti-His surface. Kinetic association (k_a) and dissociation (k_d) rate constants were determined by fitting the real-time sensorgrams to a 1:1 binding model using Scrubber v2.0c curve fitting software. Binding dissociation equilibrium constants (K_D) and dissociative half-lives ($t^{1}/_{2}$) were calculated from the kinetic rate constants as:

$$K_D\ (M) = \frac{\boldsymbol{kd}}{\boldsymbol{ka}}, \quad \text{and} \quad t^{1}/_{2}\ (min) = \frac{\boldsymbol{\ln(2)}}{\boldsymbol{60{*}kd}}$$

[0291] Anti-hCACNG1 antibodies kinetic results are presented in Table 2. As shown in table 2, all of the antibodies bound to surface-captured human CACNG1 nanodisc with several antibodies binding with single digit nM or triple digit pM affinities.

Table 2: Kinetic and Equilibrium Binding Parameters of anti-hCACNG1 antibodies to Surface-captured human CACNG1 nanodisc at 25°C

	CACNG1 nanodisc Captured (RU)	**mAb Bound at 300 nM (RU)**	**ka (1/Ms)**	**kd (1/s)**	**KD (M)**	**t1/2 (min)**
anti-hCACNG1 Ab 18/ anti-hCACNG1 Ab 2	122 ± 0.7	25	7.50E+05	5.59E-04	7.45E-10	20.7
anti-hCACNG1 Ab 3	112 ± 0.6	16	2.31E+05	3.95E-03	1.71E-08	2.9
anti-hCACNG1 Ab 20/ anti-hCACNG1 Ab 1	140 ± 1.3	14	1.04E+05	8.40E-03	8.10E-08	1.4
anti-hCACNG1 Ab 4	126 ± 0.9	19	3.42E+05	4.06E-04	1.19E-09	28.4
anti-hCACNG1 Ab 11	209 ± 1.9	23	1.93E+06	2.43E-02	1.26E-08	0.5
anti-hCACNG1 Ab 12	191 ± 3.3	22	3.31E+05	1.37E-03	4.15E-09	8.4
anti-hCACNG1 Ab 13	167 ± 1	53	7.42E+04	4.72E-05	6.35E-10	245.0
anti-hCACNG1 Ab 14	159 ± 1.5	31	1.52E+05	3.01E-04	1.98E-09	38.4
anti-hCACNG1 Ab 15	152 ± 1.9	31	7.30E+05	8.36E-05	1.14E-10	138.2
anti-hCACNG1 Ab 16	148 ± 1.9	7	2.94E+05	3.04E-02	1.04E-07	0.4
anti-hCACNG1 Ab 17	139 ± 0.6	9	1.36E+05	1.27E-02	9.35E-08	0.9

Example 3 : In vitro and ex vivo screening of purified CACNG1 antibodies using human and mouse myotubes

[0292] A total of 43 purified CACNG1 antibodies from two immunization campaigns were screened in vitro using human and mouse myotubes. Incubation of live myotubes with CACNG1 antibodies followed by fluorophore-conjugated secondary detection was performed to assess antibody binding. Live staining of human myotubes with 25nM of anti-CACNG1 antibody followed by fluorophore-conjugated secondary antibody detection displayed robust binding compared to isotype control. Incubation of myotubes with CACNG1 antibodies followed by duocarmycin-conjugated secondary was performed to assess antibody internalization via cell kill.

[0293] Immunostaining for CACNG1 in CACNG1Hu/Hu mouse single myofibers and muscle tissue cross sections confirmed that CACNG1 is expressed at the cell surface of the myofiber.

Example 4: **Binding of anti-hCACNG1 monoclonal antibodies to mouse or human myotubes, and effect of binding on calcium flux by human myoblasts**

[0294] CACNG1 is the γ1 subunit of the skeletal muscle specific L-type calcium channel, (dihydropyridine receptor), though genetic deletion of CACNG1 appears to have no major impact on skeletal muscle function. To determine whether antibodies that bind CACNG1 impact muscle function, a calcium flux assay was performed on human myotubes that were incubated with CACNG1 antibodies to determine whether these antibodies affect acetylcholine-induced calcium release.

[0295] Human skeletal myoblasts (Cook Myosite, Inc.) were plated at 10,000 cells/well of a 96-well plate and were differentiated for 7 days into myotubes. On the final day of differentiation, media was replaced with 50μL of FLIPR calcium 5 dye with probenecid (Invitrogen) and 50uL of assay buffer (0.1% BSA-DMEM) per well. CACNG1 and isotype control antibodies were serial diluted in assay buffer and added to the cells and incubated at 37°C in a 5% CO_2 incubator for 1 hour prior to the calcium flux assay. Nicardipine hydrochloride (Sigma) was added to untreated wells to serve as a control for calcium channel blockade. After 1 hour, acetylcholine (Sigma) was added at a 20uM final concentration to induce myotube calcium release, and plates were assayed on a FLIPR Tetra (Molecular Devices, LLC).

[0296] As expected, nicardipine substantially reduced human myotube calcium release, while none of the CACNG1 antibodies or isotype control antibodies had any major impact on calcium flux (Figure 1). Overall, this demonstrates that anti-hCACNG1 antibodies bind, and are internalized, but do not block calcium release in human myotubes cultured *in vitro*.

Example 4: **CACNG1 antibody binding and internalization in myofibers ex vivo**

[0297] Following confirmation of CACNG1 antibody binding to human myotubes, a subset of antibodies was tested for binding to fully mature myofibers *ex vivo*. Single myofibers were isolated from either wildtype mice, mice that were homozygous for the deletion of CACNG1 (referred to as CACNG1 knockout mice), or mice that were homozygous for the expression of human CACNG1 in place of mouse CACNG1 (referred to as CACNG1Hu/Hu). The gastrocnemius muscle was

removed, collagenase digested, and single myofibers were isolated, washed, and incubated overnight at 37°C at 5% CO2, in DMEM + 10% horse serum. Following overnight incubation, single myofibers were incubated with 100nM of each CACNG1 antibody or an isotype control antibody for 30 minutes. Myofibers were then washed twice in DMEM + 10% horse serum, and subsequently incubated with 10ug/mL of fluorescent-conjugated secondary antibodies for 30 minutes, washed twice in DMEM + 10% horse serum, and then fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Single fibers were then washed twice with PBS, stained with Hoechst for 5 minutes, washed once more with PBS, then transferred to microscope slides, coverslipped, and imaged using a Zeiss LSM 710 confocal microscope.

[0298] Separately, to determine whether an anti-human CACNG1 antibody as described herein can internalize in myofibers *ex vivo*, Alexa 647 (A647) fluorophore was directly conjugated to the CACNG1 antibody and an isotype control antibody. Single myofibers were isolated, washed, and incubated overnight. The following day, myofibers were incubated with 100nM of the A647-conjugated antibody for 30 minutes, 4 hours, or 8 hours, and were then washed twice with PBS. Myofibers were then fixed with 4% PFA for 15 minutes, stained with Hoechst for 5 minutes, washed once more with PBS, then transferred to microscope slides, coverslipped, and imaged using a Zeiss LSM 710 confocal microscope.

[0299] Two CACNG1 antibodies, anti-hCACNG1 Ab 4 and anti-hCACNG1 Ab 1, demonstrated binding to CACNG1Hu/Hu myofibers *ex vivo* and did not bind to wildtype or CACNG1 knockout myofibers. Isotype control antibodies did not bind to either CACNG1Hu/Hu myofibers or wildtype myofibers. (Figure 3).

[0300] Single plane confocal imaging revealed that the fluorophore-conjugated CACNG1 antibody, anti-hCACNG1 Ab 2, bound to the surface of the myofiber following 30 minutes of incubation, and that it was internalized within the myofiber by as early as 4 hours. (Figure 1)

[0301] In conclusion, CACNG1 antibodies can bind to the surface of single myofibers *ex vivo*. Additionally, tracking of a fluorophore conjugated CACNG1 antibody demonstrates initial binding to the surface of the myofiber, followed by internalization into the myofiber after several hours.

Example 5: **CACNG1 antibody-DHT conjugate androgen reporter assay**

[0302] CACNG1 is the γ1 subunit of the dihydropyridine receptor that is expressed specifically in skeletal muscle. Therefore, antibodies generated against CACNG1 could be used to deliver conjugated therapeutic payloads specifically to skeletal muscle to enhance therapeutic efficacy in muscle and reduce off-target toxicity. For example, conjugation of the potent metabolite of testosterone, dihydrotestosterone (DHT), to CACNG1 antibodies, may allow for androgen receptor signaling in muscle, leading to increased muscle mass and function. Here, CACNG1 antibodies conjugated to a linker with DHT payload were tested in an androgen receptor (AR) reporter cell line to determine whether these antibody conjugates can specifically activate the AR in CACNG1-expressing cells *in vitro*.

[0303] To assess signaling via the AR, LNCaP cells were transfected with lentivirus (Qiagen; ARE.Luc Cignal Lenti) to generate a stable cell line that expressed AR-luciferase reporter (AR.Luc). A subset of these selected cells was transduced to express human CACNG1 and further selected, with this cell line being referred to as hCACNG1.AR.Luc.

[0304] For the bioassay, AR.Luc or hCACNG1.AR.Luc cells were plated at 5,000 cells/well in OptiMEM and 0.5% charcoal-stripped FBS in PDL-coated 96-well plates. Cells were then incubated for 24, 48, or 27 hours with CACNG1 antibodies or isotype control antibody conjugated to DHT (via L2 linker-payload), or unconjugated DHT alone. All antibodies were conjugated with DHT at a drug-antibody-ratio (DAR) of ~4. After the respective timepoints, cells were lysed and incubated with One-GLO buffer, and luminescence was read on an Envision plate reader. Relative luminescence units (RLU) were plotted against log concentration in mol/L, adjusted for DAR.

[0305] Unconjugated DHT activated AR in both AR.Luc (Figure 2) and hCACNG1.AR.Luc cell lines (Figure 3), whereas DHT conjugated to an isotype control antibody (Isotype control Ab 1-L2) did not activate AR in either of these cell lines. Several CACNG1 antibody-DHT conjugates activated AR only in the hCACNG1.AR.Luc cell line (Figure 3), but not in the AR.Luc cell line (Figure 2). While the efficacy and potency of AR activation of CACNG1 antibody-DHT conjugates was lower than that of unconjugated DHT at 24 hours following treatment, activation of the AR was sustained at 48 and 72 hours with these conjugates, whereas unconjugated AR signal decreased substantially at these timepoints. Overall, these data demonstrate that conjugation of DHT to CACNG1 antibodies allows for specific activation of the AR in cells expressing hCACNG1, and

that DHT conjugated to CACNG1 antibodies maintains sustained AR signaling over several days in hCACNG1-expressing cells *in vitro*.

***Example 6: In vivo* biodistribution**

[0306] To determine whether CACNG1 antibodies can specifically target skeletal muscle *in vivo*, CACNG1 antibodies (anti-hCACNG1 Ab 1 and anti-hCACNG1 Ab 2) and an isotype control antibody (Isotype control Ab 4) were conjugated with Alexa Fluor 647 fluorescent dye, and were tail vein injected into mice (n = 1/group) that were homozygous for the expression of human CACNG1 in place of mouse CACNG1 (referred to as CACNG1Hu/Hu) at a dose of 10mg/kg, or with saline control. Six days following injection, mice were cryopreserved for whole body antibody distribution analysis using cryo-fluorescence tomography (Invicro). A separate set of mice (n = 1/group) that was injected with the same antibodies (or saline control) were sacrificed at 6 days post-injection and PBS perfused, and the following tissues were harvested for immunofluorescence analysis: tibialis anterior, gastrocnemius/plantaris/soleus complex, diaphragm, tongue, pelvic floor muscle, triceps, trapezius, liver, kidney, spleen, brown adipose. Tissues were embedded in optical coherence tomography (OCT) compound, frozen in liquid nitrogen-cooled isopentane, and subsequently cryo-sectioned at 10um onto microscope slides. Tissue sections were then permeabilized with Triton X-100, blocked with 4% BSA, and incubated with a rabbit-derived laminin antibody (Sigma) overnight. The following day, sections were washed, stained with anti-rabbit Alexa 488 secondary antibody (Thermo Fisher), counterstained with Hoescht, washed, fixed with 4% PFA, washed, and mounted with Fluoromount-G (Thermo Fisher). Tissues were then imaged on a Zeiss Axioscan Z1 slide scanner to visualize tissue distribution of Alexa 647-conjugated antibodies.

[0307] Alexa 647-conjugated CACNG1 antibodies, anti-hCACNG1 Ab 2 and anti-hCACNG1 Ab 1 displayed clear signal in multiple skeletal muscles via cryo-fluorescence tomography imaging, while the isotype control antibody did not show muscle uptake and accumulated mostly in the bladder (Figure 4). Saline-dosed control did not show any appreciable fluorescent signal throughout the mouse. Fluorescent signal in the muscle appeared stronger in the mouse dosed with CACNG1 antibody anti-hCACNG1 Ab 2 compared to anti-hCACNG1 Ab 1, though their overall muscle distribution pattern was similar.

[0308] Fluorescence imaging of tissue sections revealed uptake of Alexa 647-conjugated CACNG1 antibodies anti-hCACNG1 Ab 2 and anti-hCACNG1 Ab 1 in multiple skeletal muscles, including: gastrocnemius/plantaris/soleus complex, diaphragm, tongue, pelvic floor muscle, triceps, and trapezius (Figure 5). Similar to cryo-fluorescence tomography imaging findings, the overall signal of anti-hCACNG1 Ab 2 appeared stronger in the muscle sections compared to anti-hCACNG1 Ab 1. Neither anti-hCACNG1 Ab 2 and anti-hCACNG1 Ab 1 showed any clear staining in multiple non-muscle tissues, including: liver, kidney, spleen, and brown adipose (Figure 6).

[0309] These *in vivo* biodistribution studies demonstrated that fluorophore-conjugated CACNG1 antibodies specifically target skeletal muscle, and are not taken up by other non-muscle tissues.

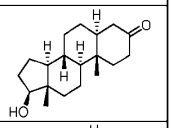
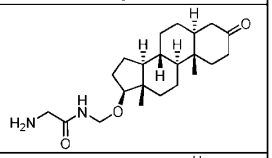
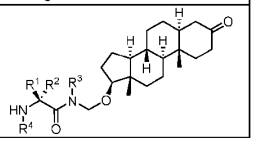
***Example 7*: CACNG1 antibody distribution to muscle is altered by exercise and dose**

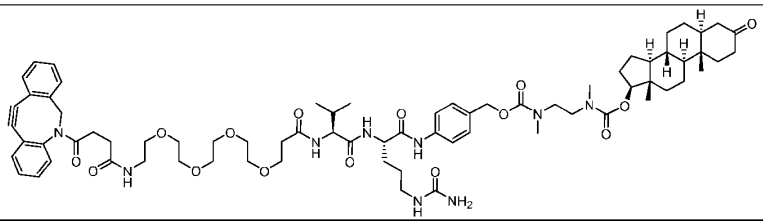
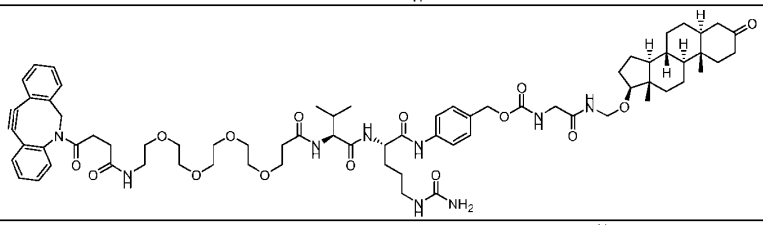
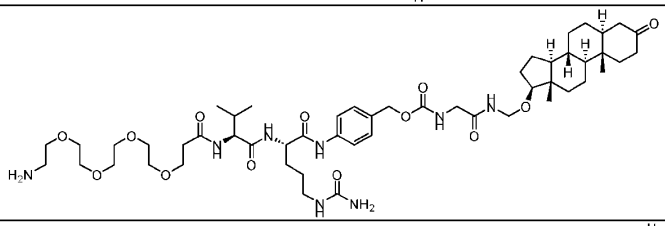
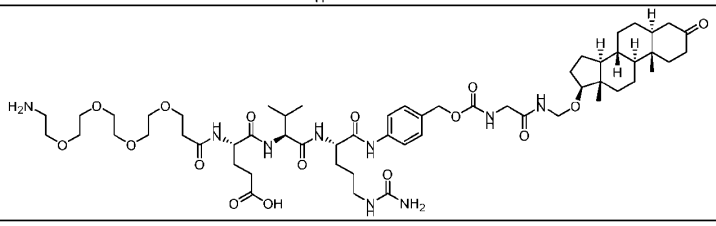
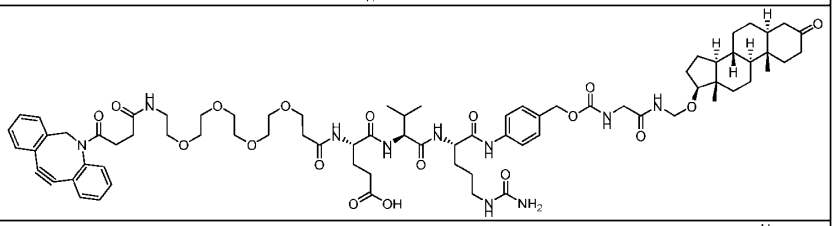
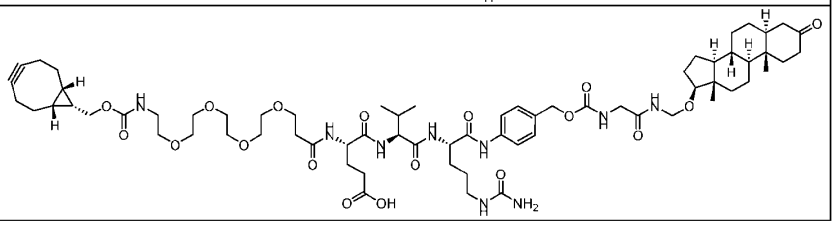
[0310] To test methods to enhance CACNG1 antibody distribution to muscle, CACNG1$^{Hu/Hu}$ mice were dosed with 10mg/kg or 50mg/kg CACNG1 antibody, and a subset of mice were given access to exercise wheels (Figure 7, top panel). Wheel running enhanced CACNG1 antibody distribution to the working soleus muscle, and 50mg/kg dose also showed enhanced distribution throughout the soleus (Figure 7, bottom panel).

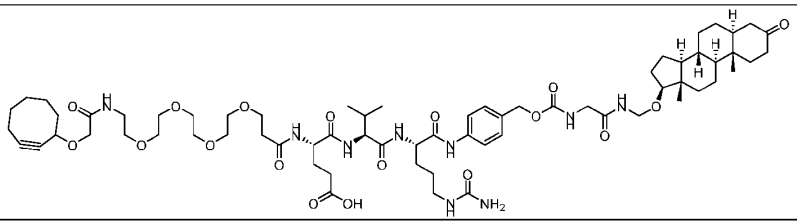
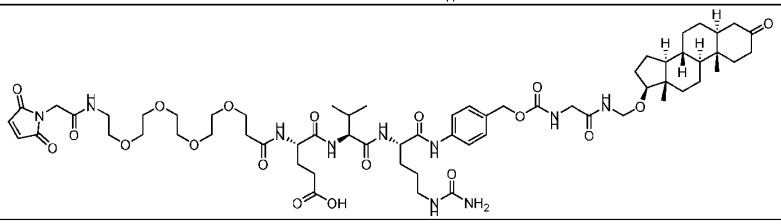
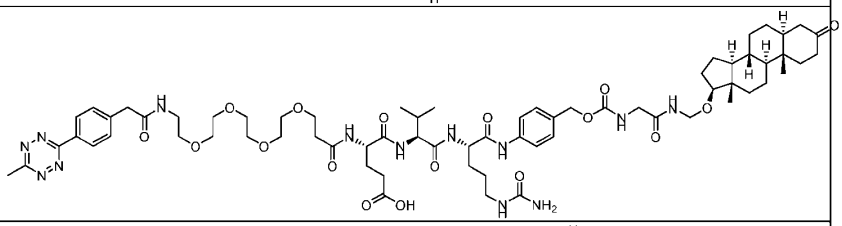
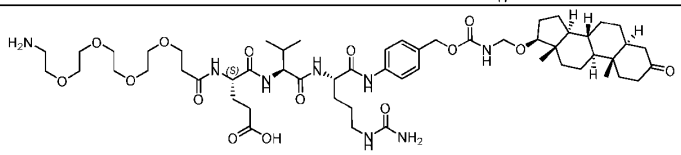
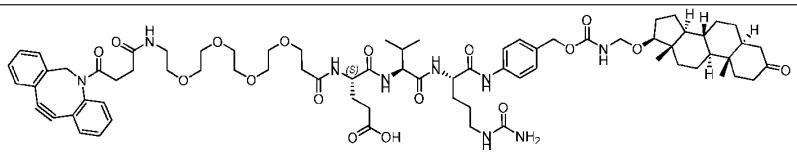
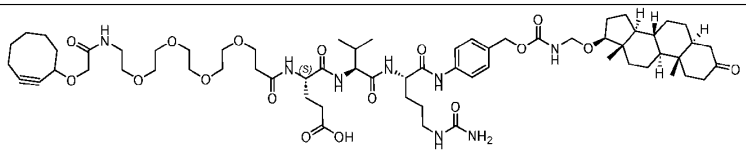
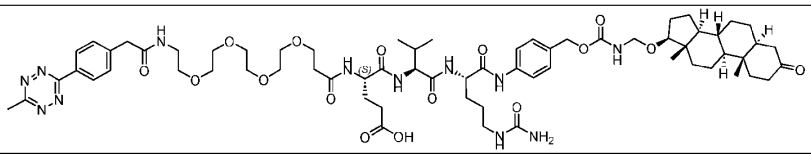
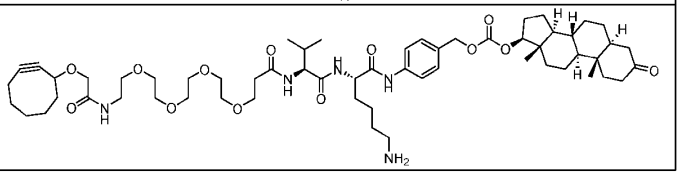
Example 8: Preparation of Payloads and Linker-Payloads

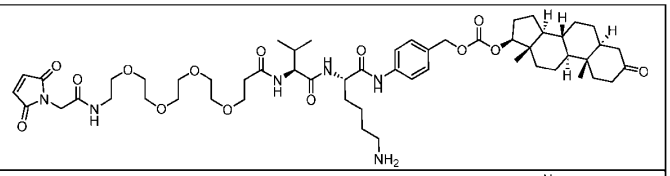
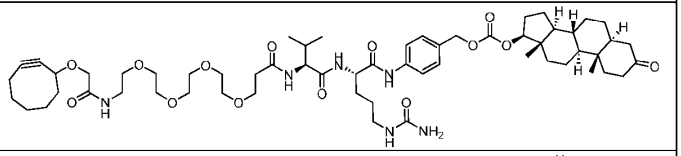
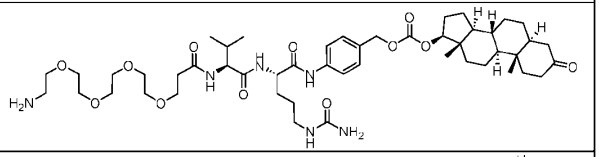
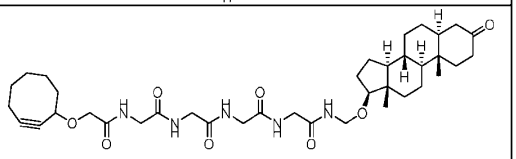
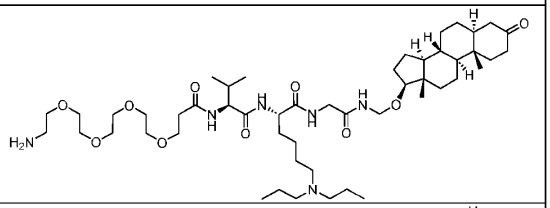
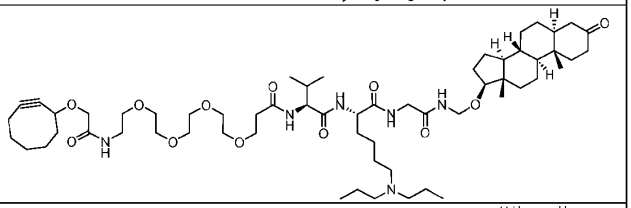
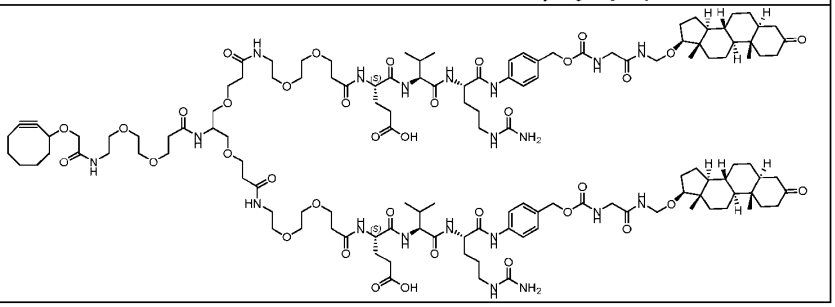
[0311] The exemplary compounds (payloads and linker-payloads) according to the present disclosure are depicted in Table 3, below.

Table 3. Structures of compounds

Cpd#	Linker	Structures
DHT	/	
ProDHT	Gly-NHCH$_2$-DHT	
Other AA-ProDHT	AA-NRCH$_2$-DHT	

LP1	DIBAC-PEG$_4$-vcPAB-DME-DHT	
LP2	DIBAC-PEG$_4$-vcPAB-ProDHT	
LP2A	NH_2-PEG$_4$-vcPAB-ProDHT	
LP3A	NH_2-PEG$_4$-EvcPAB-ProDHT	
LP3	DIBAC-PEG$_4$-EvcPAB-ProDHT	
LP31	BCN-PEG$_4$-EvcPAB-ProDHT	

LP32	COT-PEG$_4$-EvcPAB-ProDHT	
LP33	Mc-PEG$_4$-EvcPAB-ProDHT	
LP4	Tz-PEG$_4$-EvcPAB-ProDHT	
LP5A	NH_2-PEG$_4$-EvcPAB-$NHCH_2$-DHT	
LP5	DIBAC-PEG$_4$-EvcPAB-$NHCH_2$-DHT	
LP51	COT-PEG$_4$-EvcPAB-$NHCH_2$-DHT	
LP52	Tz-PEG$_4$-EvcPAB-$NHCH_2$-DHT	
LP41	COT-PEG$_4$-VK-PABC-DHT	

LP42	Mc-PEG$_4$-VK-PABC-DHT	
LP43	COT-PEG$_4$-vcPABC-DHT	
LP44	NH_2-PEG$_4$-vcPABC-DHT	
LP6	COT-GGGG-$NHCH_2$-DHT (SEQ ID NO: 292)	(SEQ ID NO: 292) 
LP61A	NH_2-PEG$_4$-VK(Pr_2)G-$NHCH_2$-DHT	
LP61	COT-PEG$_4$-VK(Pr_2)G-$NHCH_2$-DHT	
LP7	COT-PEG4-branch-PEG2-EvcPAB-ProDHT	

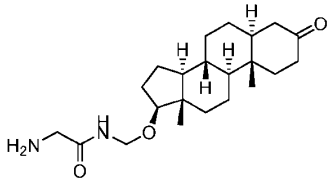
[0312] Chemical properties of the exemplary compounds (payloads and linker-payloads) according to the present disclosure are depicted in Table 4, below.

Table 4. Chemical Properties of Payloads and Linker-payloads:

Cpd#	Linker-name	cLogP	MF	MW	HPLC purity (%)	HPLC RT (min)	Mass ESI m/z
ProDHT	Gly-$NHCH_2$-DHT	2.26	$C_{22}H_{36}N_2O_3$	376.5	>99 (LCMS)	1.03 (LCMS)	399 $(M + Na)^+$
LP1	DIBAC-PEG_4-vcPAB-DME-DHT	5.46	$C_{73}H_{101}N_9O_{15}$	1344.7	>99	8.31	672.9 $(M/2 + H)^+$
LP2	DIBAC-PEG_4-vcPAB-ProDHT	4.33	$C_{71}H_{97}N_9O_{15}$	1316.6	>99	8.40	659 $(M/2 + H)^+$
LP2A	NH_2-PEG_4-vcPAB-ProDHT	1.51	$C_{52}H_{84}N_8O_{13}$	1029.3	>90		1029.6 $(M + H)^+$
LP3A	NH_2-PEG_4-EvcPAB-ProDHT	-1.64	$C_{57}H_{91}N_9O_{16}$	1158.4	>93 (LCMS)	1.36 (LCMS)	1160 $(M + H)^+$
LP3	DIBAC-PEG_4-EvcPAB-ProDHT	3.44	$C_{76}H_{104}N_{10}O_{18}$	1445.7	>99	7.32	723 $(M/2 + H)^+$
LP31	BCN-PEG_4-EvcPAB-ProDHT	3.31	$C_{68}H_{103}N_9O_{18}$	1334.6	96		668 $(M/2 + H)^+$
LP32	COT-PEG_4-EvcPAB-ProDHT	2.69	$C_{67}H_{103}N_9O_{18}$	1322.6	97		662 $(M/2 + H)^+$.
LP33	Mc-PEG_4-EvcPAB-ProDHT	-0.27	$C_{63}H_{94}N_{10}O_{19}$	1295.5	>90		648.3 $(M/2 + H)^+$
LP4	Tz-PEG_4-EvcPAB-ProDHT	1.78	$C_{68}H_{99}N_{13}O_{17}$	1370.6	>98	8.74	1371 $(M + H)^+$
LP5A	NH_2-PEG_4-EvcPAB-$NHCH_2O$-DHT	-0.54	$C_{55}H_{88}N_8O_{15}$	1101.4	>98	6.90	1101 $(M + H)^+$
LP5	DIBAC-PEG_4-EvcPAB-$NHCH_2O$-DHT	4.54	$C_{74}H_{101}N_9O_{17}$	1388.7	>99	7.24	695 $(M/2 + H)^+$
LP51	COT-PEG_4-EvcPAB-$NHCH_2$-DHT	3.80	$C_{65}H_{100}N_8O_{17}$	1265.6	96		632.3 $(M/2 + H)^+$
LP52	Tz-PEG_4-EvcPAB-$NHCH_2$-DHT	2.81	$C_{66}H_{96}N_{12}O_{16}$	1313.6	>90		657.3 $(M/2 + H)^+$
LP41	COT-PEG_4-VK-PABC-DHT	6.32	$C_{59}H_{91}N_5O_{13}$	1078.4	>95		1079.5 $(M + H)^+$
LP42	Mc-PEG_4-VK-PABC-DHT	3.35	$C_{55}H_{82}N_6O_{14}$	1051.3	>98		1052 $(M + H)^+$
LP43	COT-PEG_4-vcPABC-DHT	5.36	$C_{59}H_{90}N_6O_{14}$	1107.4	>95		1108 $(M + H)^+$
LP44	NH_2-PEG_4-vcPABC-DHT	3.28	$C_{49}H_{78}N_6O_{12}$	943.2	>90		943.5 $(M + H)^+$
LP6	COT-GGGG-$NHCH_2$-DHT (SEQ ID NO: 292)	1.02	$C_{38}H_{57}N_5O_8$	711.9	>99	7.99	422 $(M_{LP6} - M_{DHT} + H)^+$

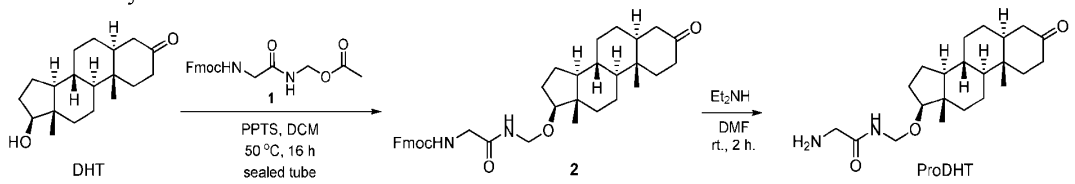
LP61A	NH_2-PEG4-VK(Pr_2)G-$NHCH_2$-DHT	3.18	$C_{50}H_{90}N_6O_{10}$	935.9	>90		935.7 $(M + H)^+$
LP61	COT-PEG4-VK(Pr_2)G-$NHCH_2$-DHT	5.26	$C_{60}H_{102}N_6O_{12}$	1099.5	>90		1100.0 $(M + H)^+$
LP7	COT-PEG4-branch-PEG2-EvcPAB-ProDHT	1.40	$C_{132}H_{204}N_{20}O_{37}$	2663.2	>98	7.50	1332 $(M/2 + H)^+$

ProDHT: 2-amino-*N*-({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo [8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)acetamide (ProDHT)

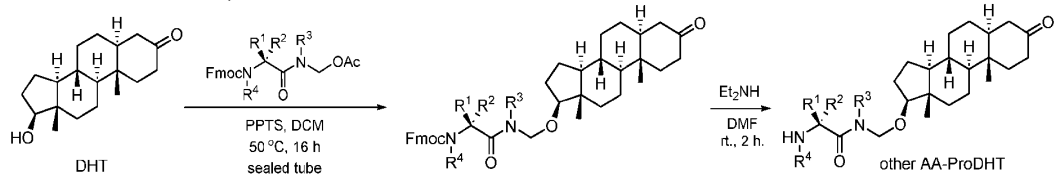


[0313] Synthesis of ProDHT and other AA-ProDHT is depicted in Schemes 1 and 1a, below.

Scheme 1. Synthesis of ProDHT



Scheme 1a. Generic Synthesis of other AA ProDHT



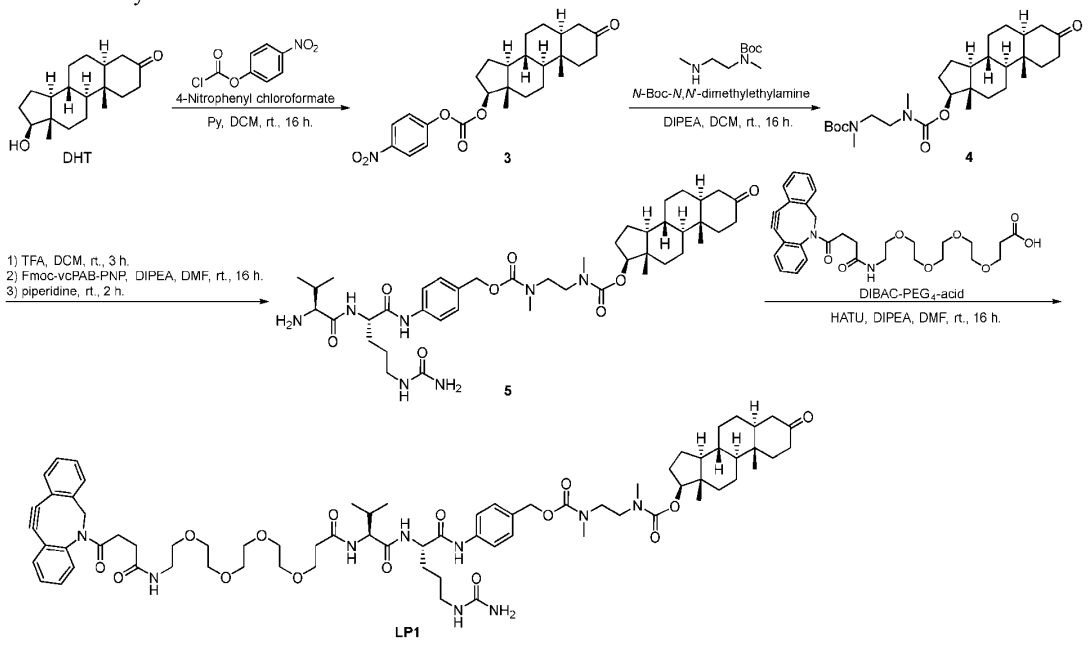
[0314] A sealed tube was charged with DHT (0.40 g, 1.4 mmol), compound **1** (0.51 g, 1.4 mmol), PPTS (36 mg, 0.14 mmol) and DCM (8 mL). The tube was sealed and the reaction mixture was stirred at 50 °C for 16 hours, which was monitored by LCMS. The resulting mixture was directly separated by prep-HPLC (5-95% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give compound **2** (0.52 g, ESI m/z 621 $(M + Na)^+$) as a white solid, which was dissolved in DMF (5 mL). To the solution was added diethylamine (0.32 g, 4.3 mmol), and the reaction mixture was stirred at room temperature for 2 hours until Fmoc was totally removed according to LCMS. The

resulting mixture was directly separated by reversed phase flash chromatography (5-95% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give ProDHT (0.30 g, 58% yield) as a white solid. ESI m/z 399 $(M + Na)^+$. ^1H NMR (400 MHz, $DMSO_{d6}$) δ 8.60-8.41 (m, 1H), 4.69-4.48 (m, 2H), 3.57-3.39 (m, 4H), 3.14 (s, 1H), 2.46-2.36 (m, 1H), 2.34-2.25 (m, 1H), 2.12-1.82 (m, 4H), 1.79-1.71 (m, 1H), 1.65-1.58 (m, 1H), 1.56-1.03 (m, 12H), 0.97 (s, 3H), 0.94-0.63 (m, 2H), 0.63 (s, 3H) ppm.

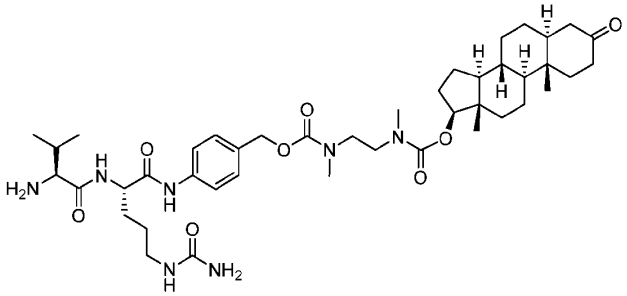
LP1

[0315] Synthesis of LP1 is depicted in Scheme 2, below.

Scheme 2. Synthesis of LP1



{4-[(2*S*)-2-[(2*S*)-2-amino-3-methylbutanamido]-5-(carbamoylamino)pentanamido] phenyl}methyl *N*-{2-[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo [8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}carbonyl)(methyl)amino]ethyl}-*N*-methylcarbamate (5)



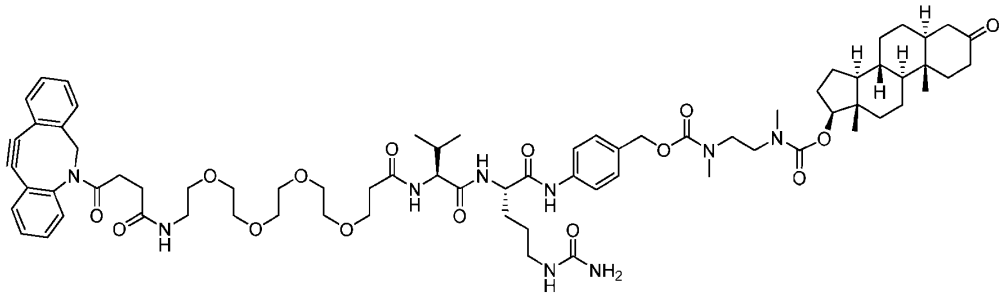
[0316] A mixture of DHT (1.2 g, 4.1 mmol) and 4-nitrophenyl chloroformate (0.89 g, 4.4 mmol) in DCM (15 mL) was cooled to 0 °C, and at this temperature pyridine (0.38 g, 4.8 mmol) was then added into the mixture. The reaction mixture was stirred at room temperature for 16 hours, which was monitored by LCMS. The resulting mixture was diluted with DCM (150 mL), washed by *sat. aq.* sodium bicarbonate (30 mL) and brine, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The residue was purified by silica gel flash chromatography (30-100% DCM in petroleum ether) to give compound **3** (1.6 g) as an off-white solid.

[0317] To a solution of compound **3** (0.91 g, 2.0 mmol) in DCM (10 mL) were added *N*-Boc-*N*,*N*'-dimethylethylamine (0.75 g, 4.0 mmol) and DIPEA (0.52 g, 4.0 mmol), and the reaction mixture was stirred at room temperature for 16 hours, which was monitored by LCMS. The resulting mixture was diluted with DCM (150 mL), washed with *aq.* citric acid (1 M, 40 mL x 2), *sat. aq.* sodium bicarbonate (40 mL x 2) and brine (40 mL) successively, dried over anhydrous sodium sulfate and concentrated *in vacuo* to give compound **4** (0.50 g) as an off-white solid.

[0318] To a solution of compound **4** (0.20 g, 0.40 mmol) in DCM (5 mL) was added TFA (0.5 mL), and the mixture was stirred at room temperature for 3 hours. The resulting mixture was concentrated *in vacuo*. The residue was dissolved in DMF (3 mL), and to the solution were added Fmoc-vcPAB-PNP (CAS: 863971-53-3, 0.30 g, 0.39 mmol) and DIPEA (0.5 mL). The reaction mixture was stirred at room temperature for 16 hours, which was monitored by LCMS. To the resulting solution was then added piperidine (0.2 mL) in one portion, and the mixture was stirred at room temperature for another 2 hours until Fmoc was totally removed according to LCMS. The

mixture was directly separated by prep-HPLC (5-95% acetonitrile in *aq.* TFA (0.1%)) to give compound **5** (50 mg, 6.0% total yield from DHT) as a white solid. ESI m/z 810.3 $(M + H)^+$.

{4-[(2*S*)-2-[(2*S*)-2-[1-(4-{2-azatricyclo[10.4.0.0^{4,9}]hexadeca-1(12),4(9),5,7,13,15-hexaen-10-yn-2-yl}-4-oxobutanamido)-3,6,9,12-tetraoxapentadecan-15-amido]-3-methylbutanamido]-5-(carbamoylamino)pentanamido]phenyl}methyl *N*-{2-[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadecan-14-yl]oxy}carbonyl)(methyl)amino]ethyl}-*N*-methylcarbamate (LP1)

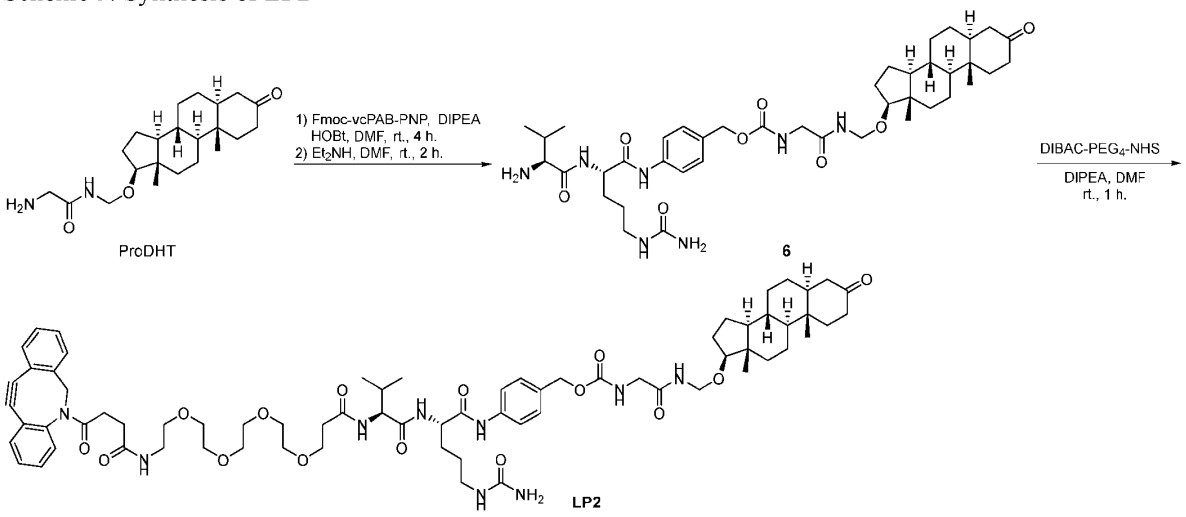


[0319] To a solution of compound **5** (0.25 g, 31 μmol) in DMF (0.55 mL) were added DIBAC-PEG4-acid (CAS: 1537170-85-6, 20 mg, 37 μmol), DIPEA (19 mg, 0.15 mmol) and HATU (17 mg, 45 μmol), and the reaction mixture was stirred at room temperature for 16 hours, which was monitored by LCMS. The resulting mixture was directly separated by prep-HPLC (5-95% acetonitrile in *aq.* bicarbonate (10 mM)) to give **LP1** (13 mg, 31% yield) as a white solid. ESI m/z 672.9 $(M/2 + H)^+$. ^{1}H NMR (400 MHz, $DMSO_{d6}$) δ 9.98 (s, 1H), 8.12 (d, J = 7.3 Hz, 1H), 7.91-7.24 (m, 13H), 5.98 (t, J = 5.5 Hz, 1H), 5.41 (s, 2H), 5.08-4.88 (m, 3H), 4.45-4.15 (m, 3H), 3.64-3.55 (m, 3H), 3.50-3.42 (m, 9H), 3.31-3.28 (m, 2H), 3.14-2.89 (m, 4H), 2.88-2.65 (m, 5H), 2.64-2.16 (m, 20H), 2.13-1.83 (m, 6H), 1.81-1.15 (m, 14H), 1.01-0.63 (m, 14H) ppm.

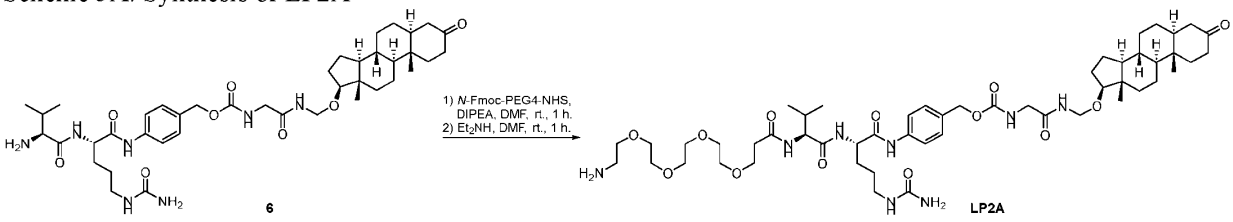
LP2 and LP2A

[0320] Synthesis of LP2 is depicted in Scheme 3, and synthesis of LP2A is depicted in Scheme 3a, below.

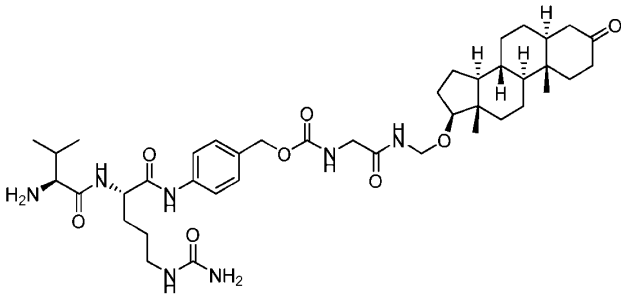
Scheme 3. Synthesis of LP2



Scheme 3A. Synthesis of LP2A



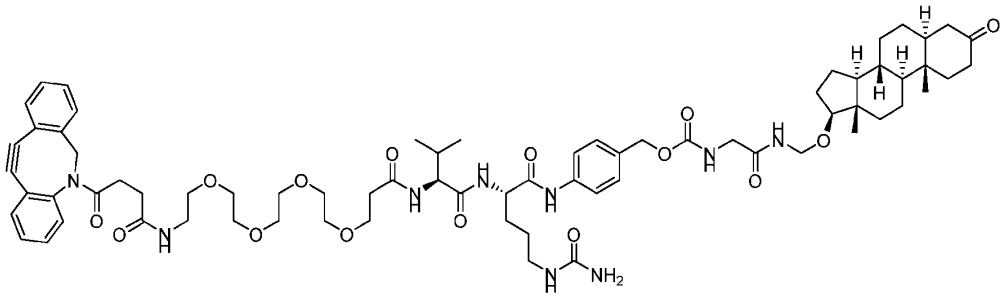
{4-[(2*S*)-2-[(2*S*)-2-amino-3-methylbutanamido]-5-(carbamoylamino)pentanamido] phenyl}methyl *N*-{[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo [8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamate (6)



[0321] To a solution of ProDHT (0.10 g, 0.27 mmol) in DMF (5 mL) were added HOBt (18 mg, 0.13 mmol) and DIPEA (0.11 g, 0.81 mmol), and the mixture was stirred at room temperature for

10 minutes before the addition of Fmoc-vcPAB-PNP (0.21 g, 0.27 mmol). The reaction mixture was stirred at room temperature for 4 hours, which was monitored by LCMS. The resulting mixture was directly separated by prep-HPLC (5-95% acetonitrile in *aq.* TFA (0.1%)) to give a white solid (0.17 g, ESI m/z 1027.5 $(M + Na)^+$), which was dissolved in DMF (5 mL). To the solution was added diethylamine (60 mg, 0.82 mmol), and the reaction mixture was stirred at room temperature for 2 hours, which was monitored by LCMS. The resulting mixture was directly purified by reversed phase flash chromatography (0-100% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give compound **6** (0.11 g, 52% yield) as a white solid. ESI m/z 783 $(M + H)^+$.

{4-[(2*S*)-2-[(2*S*)-2-[1-(4-{2-azatricyclo[10.4.0.0^{4,9}]hexadeca-1(12),4(9),5,7,13,15-hexaen-10-yn-2-yl}-4-oxobutanamido)-3,6,9,12-tetraoxapentadecan-15-amido]-3-methylbutanamido]-5-(carbamoylamino)pentanamido]phenyl}methyl *N*-{[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamate (LP2)



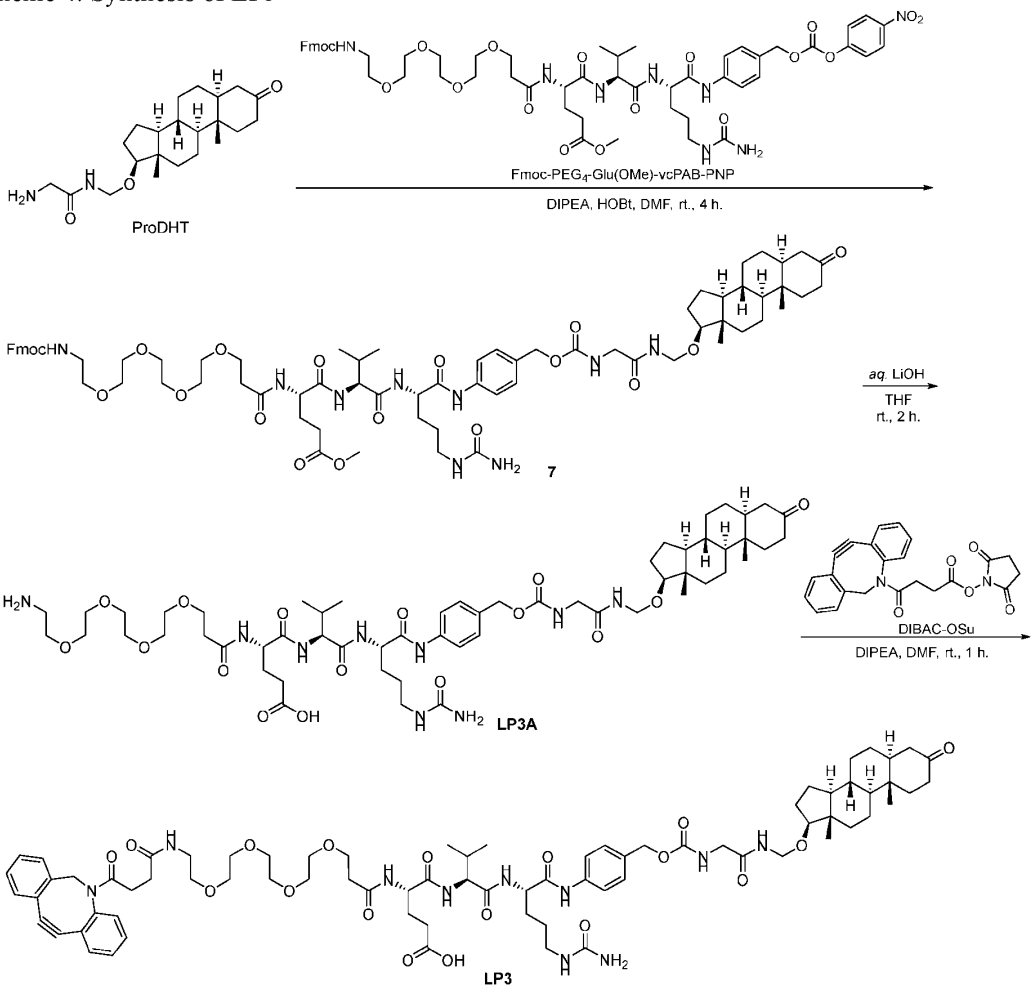
[0322] To a stirred solution of compound **6** (0.11 g, 0.14 mmol) in DMF (4 mL) were added DIBAC-PEG$_4$-NHS (0.14 g, 0.14 mmol) and DIPEA (53 mg, 0.41 mmol), and the reaction mixture was stirred at room temperature for an hour, which was monitored by LCMS. The resulting mixture was directly separated by reversed phase flash chromatography (5-95% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give **LP2** (90 mg, 51% yield) as a white solid. ESI m/z 659 $(M/2 + H)^+$. ^{1}H NMR (400 MHz, DMSO$_{d6}$) δ 10.00 (s, 1H), 8.56 (t, J = 6.7 Hz, 1H), 8.14 (d, J = 7.8 Hz, 1H), 7.89 (d, J = 8.9 Hz, 1H), 7.78 (t, J = 5.5 Hz, 1H), 7.68 (dd, J = 7.3, 1.4 Hz, 1H), 7.65-7.58 (m, 3H), 7.52-7.39 (m, 4H), 7.38-7.33 (m, 2H), 7.31-7.27 (m, 2H), 5.98 (t, J = 6.0 Hz, 1H), 5.42 (s, 2H), 5.03 (d, J = 14.0 Hz, 1H), 4.96 (s, 2H), 4.60-4.51 (m, 1H), 4.50-4.45 (m, 1H), 4.42-4.26 (m, 1H), 4.23-4.20 (m, 1H), 3.67-3.55 (m, 5H), 3.52-3.42 (m, 11H), 3.30-3.15 (m, 2H), 3.13-2.89 (m,

5H), 2.70-2.20 (m, 6H), 2.15-1.65 (m, 8H), 1.58-1.05 (m, 21H), 0.96 (s, 3H), 0.84 (d, J = 6.8 Hz, 3H), 0.82 (d, J = 6.8 Hz, 3H), 0.67 (s, 3H) ppm.

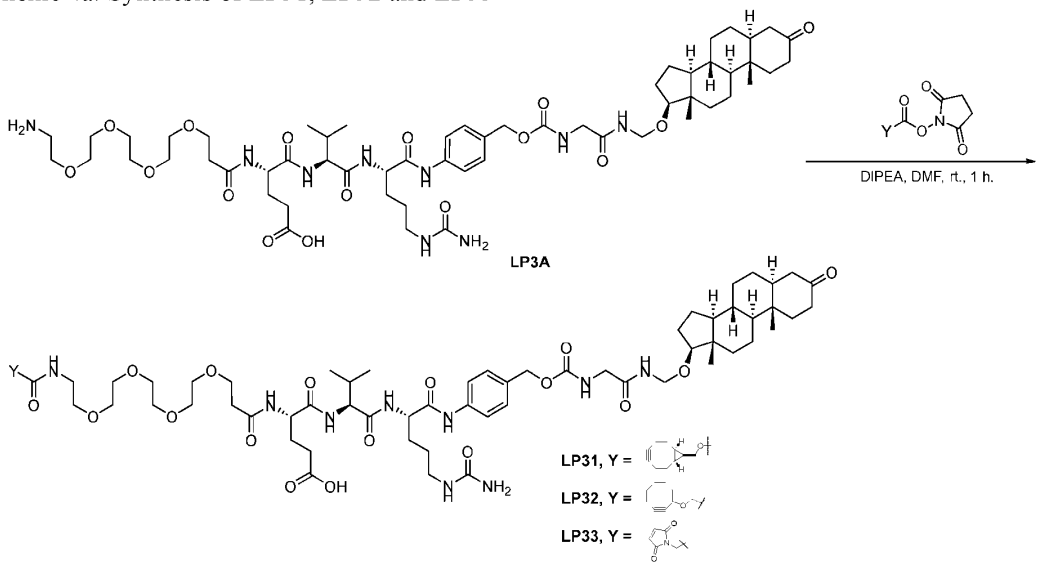
LP3A, LP3, LP31, LP32 and LP33

[0323] Synthesis of LP3A and LP3 is depicted in Scheme 4, below. Synthes if LP31, LP32 and LP33 is depicted in Scheme 4a, below.

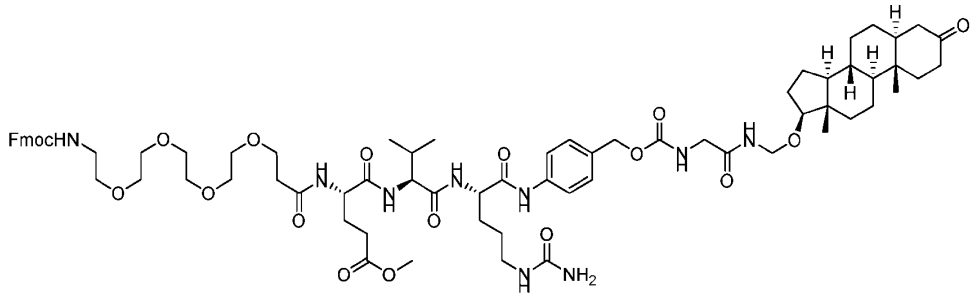
Scheme 4. Synthesis of LP3



Scheme 4a. Synthesis of LP31, LP32 and LP33

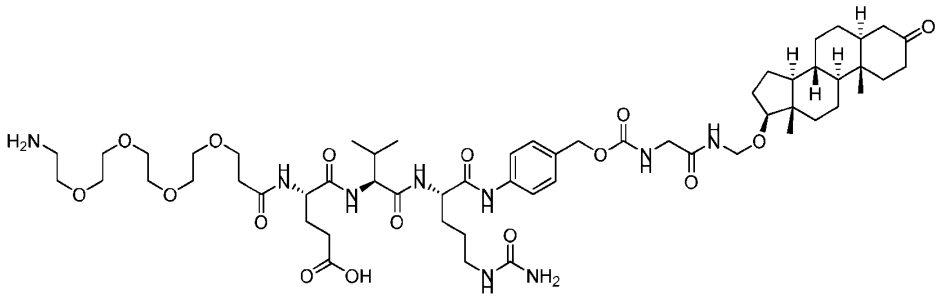


methyl (4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-[(4-{[({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}] heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl) carbamoyl] butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-[1-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-3,6,9,12-tetraoxapentadecan-15-amido]butanoate (7)



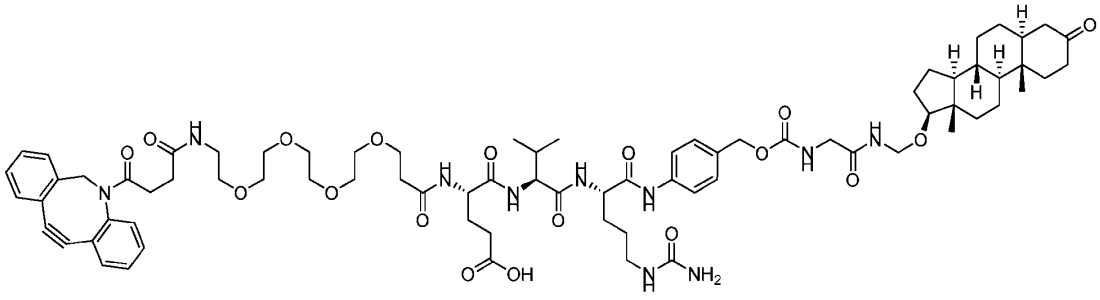
[0324] To a solution of ProDHT (35 mg, 93 μmol) in DMF (5 mL) were added HOBt (6.0 mg, 44 μmol) and DIPEA (35 mg, 0.27 mmol), and the mixture was stirred at room temperature for 10 minutes before the addition of Fmoc-PEG4-Glu(OMe)-vcPAB-PNP (CAS: 2758874-82-5, synthesized according to WO2022015656, 0.11 g, 95 μmol). The reaction mixture was stirred at room temperature for 4 hours, which was monitored by LCMS. The resulting mixture was directly separated by prep-HPLC (5-95% acetonitrile in *aq.* TFA (0.01%)) to give compound **7** (0.11 g, 85% yield) as a white solid. ESI m/z 1396 $(M + H)^+$.

(4*S*)-4-(1-amino-3,6,9,12-tetraoxapentadecan-15-amido)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-[(4-{[({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl)carbamoyl]butyl]carbamoyl}-2-methylpropyl]carbamoyl}butanoic acid (LP3A)



[0325] To a solution of compound **7** (0.11 g, 77 μmol) in THF (5 mL) was added *aq.* lithium hydroxide (1 M, 0.38 mL), and the mixture was stirred at room temperature for 2 hours, which was monitored by LCMS. The volatiles were removed *in vacuo* and the residue was separated by reversed phase flash chromatography (5-95% acetonitrile in *aq.* TFA (0.01%)) to give compound **LP3A** (78 mg, 88% yield) as a white solid. ESI m/z 1160 $(M + H)^{+}$.

(4*S*)-4-[1-(4-{2-azatricyclo[10.4.0.0^{4},^{9}]hexadeca-1(12),4(9),5,7,13,15-hexaen-10-yn-2-yl}-4-oxobutanamido)-3,6,9,12-tetraoxapentadecan-15-amido]-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-[(4-{[({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl)carbamoyl]butyl]carbamoyl}-2-methylpropyl]carbamoyl}butanoic acid (LP3)

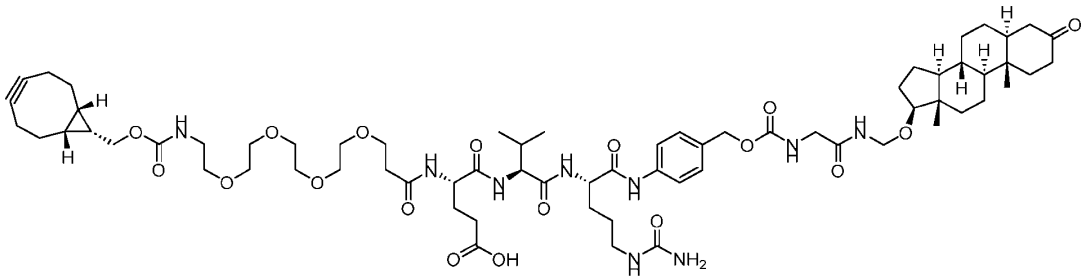


[0326] To a stirred mixture of **LP3A** (78 mg, 67 μmol) in DMF (4 mL) were added DIBAC-OSu (CAS: 1353016-71-3, commercial, 41 mg, 67 μmol) and DIPEA (26 mg, 0.20 mmol) successively,

and the reaction mixture was stirred at room temperature for an hour, which was monitored by LCMS. The resulting mixture was directly separated by prep-HPLC (5-95% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give **LP3** (30 mg, 31% yield) as a white solid. ESI m/z 723 $(M/2 + H)^+$. ^1H NMR (400 MHz, $DMSO_{d6}$) δ 10.00 (s, 1H), 8.56 (t, J = 6.7 Hz, 1H), 8.14 (d, J = 7.8 Hz, 1H), 7.89 (d, J = 8.9 Hz, 1H), 7.83-7.70 (m, 1H), 7.68 (dd, J = 7.3, 1.4 Hz, 1H), 7.65-7.55 (m, 3H), 7.52-7.25 (m, 8H), 6.01 (br s, 1H), 5.42 (s, 2H), 5.03 (d, J = 14.0 Hz, 1H), 4.96 (s, 2H), 4.60-4.51 (m, 1H), 4.50-4.45 (m, 1H), 4.42-4.26 (m, 1H), 4.23-4.20 (m, 1H), 3.67-3.55 (m, 5H), 3.52-3.20 (m, 15H), 3.13-2.89 (m, 5H), 2.70-2.40 (m, 9H), 2.30-1.84 (m, 8H), 1.80-1.05 (m, 22H), 0.96 (s, 3H), 0.85 (d, J = 6.7 Hz, 3H), 0.83 (d, J = 6.7 Hz, 3H), 0.67 (s, 3H) ppm. (COOH was not revealed.)

LP31, LP32 and LP33

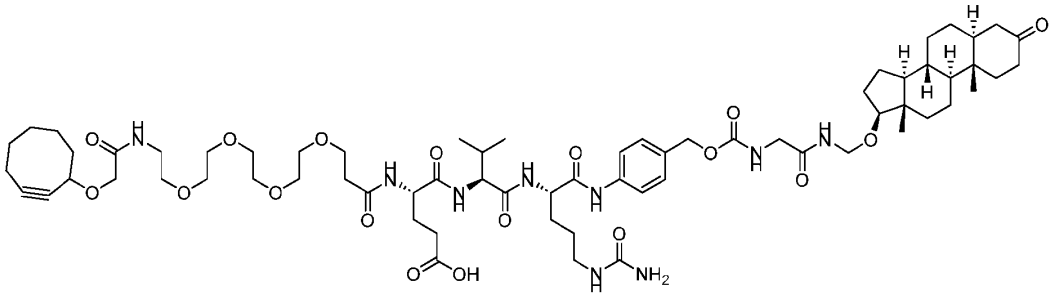
[0327] (4S)-4-{1-[({[(1R,8S,9R)-bicyclo[6.1.0]non-4-yn-9-yl]methoxy}carbonyl)amino]-3,6,9,12-tetraoxapentadecan-15-amido}-4-{[(1S)-1-{[(1S)-4-(carbamoylamino)-1-[(4-{[({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl)carbamoyl]butyl]carbamoyl}-2-methylpropyl]carbamoyl}butanoic acid (LP31)



[0328] Following a similar procedure to LP3 except using BCN-OSu instead of DIBAC-OSu, linker-payload LP31 (1.2 mg, 18% yield) was obtained as a white solid. ESI m/z: 667.9 (M/2 + H)+.

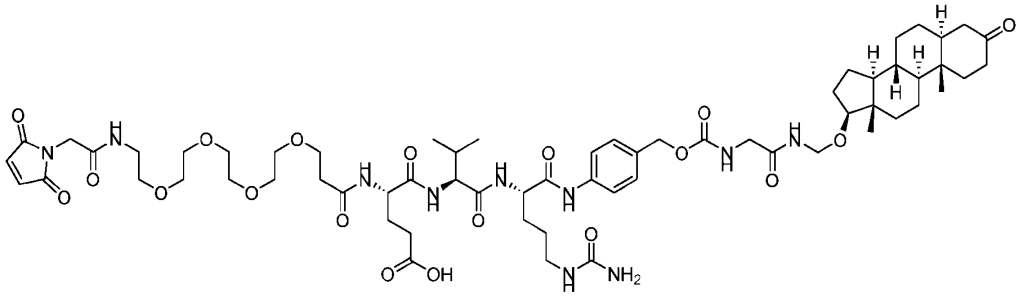
(4S)-4-{[(1S)-1-{[(1S)-4-(carbamoylamino)-1-[(4-{[({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl)carbamoyl]butyl]carbamoyl}-2-

methylpropyl]carbamoyl}-4-{1-[2-(cyclooct-2-yn-1-yloxy)acetamido]-3,6,9,12-tetraoxapentadecan-15-amido}butanoic acid (LP32)



[0329] Following a similar procedure to LP3 except using COT-OSu instead of DIBAC-OSu, linker-payload LP32 (2.2 mg, 33% yield) was obtained as a white solid. ESI m/z: 661.9 (M/2 + H)+.

(4S)-4-{[(1S)-1-{[(1S)-4-(carbamoylamino)-1-[(4-{[({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl)carbamoyl]butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-{1-[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido]-3,6,9,12-tetraoxapentadecan-15-amido}butanoic acid (LP33)

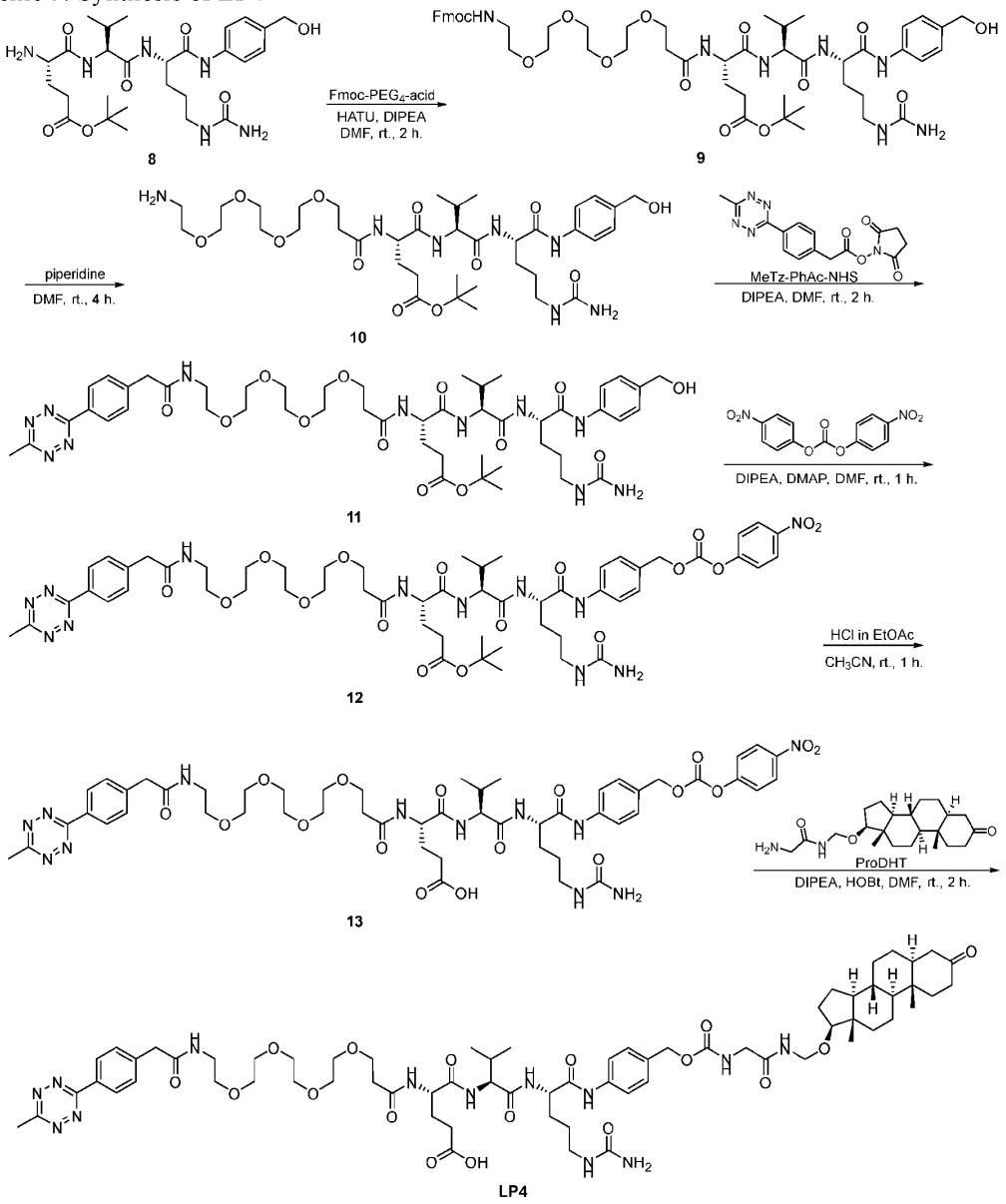


[0330] Following a similar procedure to LP3 except using AMAS instead of DIBAC-OSu, linker-payload LP33 (0.9 mg, 14% yield) was obtained as a white solid. ESI m/z: 648.3 (M/2 + H)+.

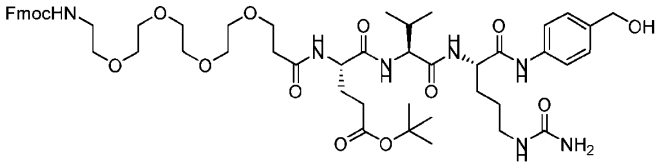
LP4

[0331] Synthesis of LP4 is depicted in Scheme 5, below.

Scheme 5. Synthesis of LP4

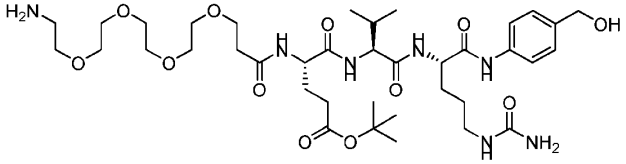


tert-butyl (4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-(hydroxymethyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-[1-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-3,6,9,12-tetraoxapentadecan-15-amido]butanoate (9)



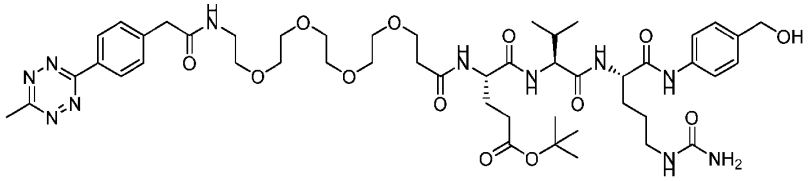
[0332] To a solution of compound Fmoc-PEG4-acid (CAS: 557756-85-1, 0.49 g, 1.0 mmol) in DMF (5 mL) were added HATU (0.38 g, 1.0 mmol) and DIPEA (0.26 g, 2.0 mmol), and the reaction mixture was stirred at room temperature for 5 minutes. To the solution was added compound **8** (CAS: 2757059-05-3, synthesized according to WO2021262910, 0.56 g, 1.0 mmol), and the reaction mixture was stirred at room temperature for 2 hours, which was monitored by LCMS. The resulting mixture was diluted with water (100 mL) and extracted with ethyl acetate (100 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated *in vacuo* to give crude compound **9**, which was purified by silica gel flash chromatography (8-10% methanol in DCM) to give compound **9** (0.50 g, 48% yield) as colorless oil. ESI m/z: 1034.3 $(M + H)^+$.

tert-butyl (4*S*)-4-(1-amino-3,6,9,12-tetraoxapentadecan-15-amido)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-(hydroxymethyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}butanoate (10)



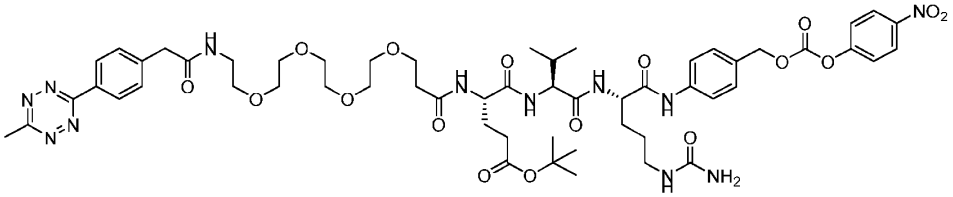
[0333] To a solution of compound **9** (0.50 g, 0.48 mmol) in DMF (5 mL) was added piperidine (82 mg, 0.96 mmol), and the reaction mixture was stirred at room temperature for 4 hours, which was monitored by LCMS. The resulting mixture was directly purified by reversed phase flash chromatography (5-95% acetonitrile in *aq.* TFA (0.03%)) to give compound **10** (0.30 g, 67% yield, TFA salt) as colorless oil. ESI m/z: 812.9 $(M + H)^+$.

tert-butyl (4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-(hydroxymethyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-(1-{2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetamido}-3,6,9,12-tetraoxapentadecan-15-amido)butanoate (11)



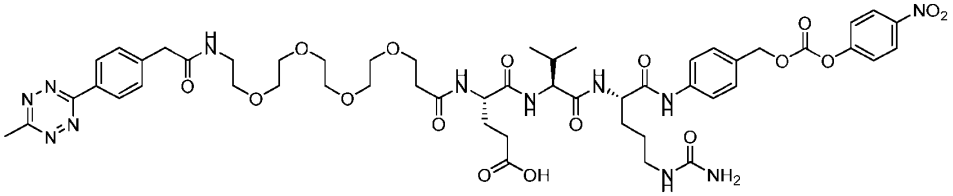
[0334] To a solution of MeTz-PhAc-NHS (CAS: 1644644-96-1, 33 mg, 0.10 mmol) in DMF (5 mL) were added compound **10** (93 mg, 0.10 mmol, TFA salt) and DIPEA (26 mg, 0.20 mmol). The reaction mixture was stirred at room temperature for 2 hours, which was monitored by LCMS. The resulting mixture was directly purified by reversed phase flash chromatography (5-95% acetonitrile in *aq.* TFA (0.03%)) to give compound **11** (80 mg, 78% yield) as a red solid. ESI m/z: 1023.5 $(M + H)^+$.

tert-butyl (4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-({[(4-nitrophenoxy)carbonyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-(1-{2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetamido}-3,6,9,12-tetraoxapentadecan-15-amido)butanoate (12)



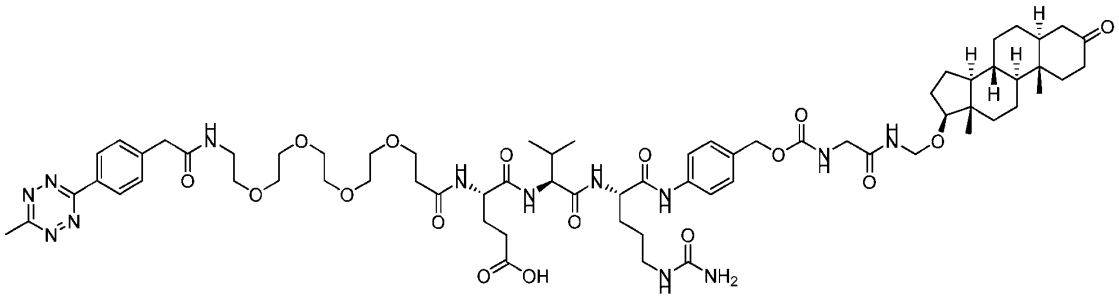
[0335] To a solution of compound **11** (0.10 g, 98 μmol) in DMF (5 mL) were added DIPEA (26 mg, 0.20 mmol), DMAP (12 mg, 98 μmol) and bis(4-nitrophenyl) carbonate (0.12 g, 0.40 mmol), and the reaction mixture was stirred at room temperature for an hour, which was monitored by LCMS. The resulting mixture was directly separated by reversed phase flash chromatography (5-95% acetonitrile in *aq.* TFA (0.03%)) to give compound **12** (60 mg, 51% yield) as a red solid. ESI m/z: 1189.5 $(M + H)^+$.

(4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-({[(4-nitrophenoxy)carbonyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-(1-{2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetamido}-3,6,9,12-tetraoxapentadecan-15-amido)butanoic acid (13)



[0336] To a solution of compound **12** (60 mg, 51 μmol) in acetonitrile (2.5 mL) was added a solution of HCl in ethyl acetate (4 N, 2.5 mL). The reaction mixture was stirred at room temperature for an hour, which was monitored by LCMS. The reaction mixture was quenched with *sat. aq.* sodium bicarbonate until pH 6-7. The organic layer was separated, dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by reversed phase flash chromatography (0-100% acetonitrile in *aq.* TFA (0.03%)) to give compound **13** (30 mg, 51% yield) as a red solid. ESI m/z: 1133.5 $(M + H)^+$.

(4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-[(4-{[({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl)carbamoyl]butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-(1-{2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetamido}-3,6,9,12-tetraoxapentadecan-15-amido)butanoic acid (LP4)



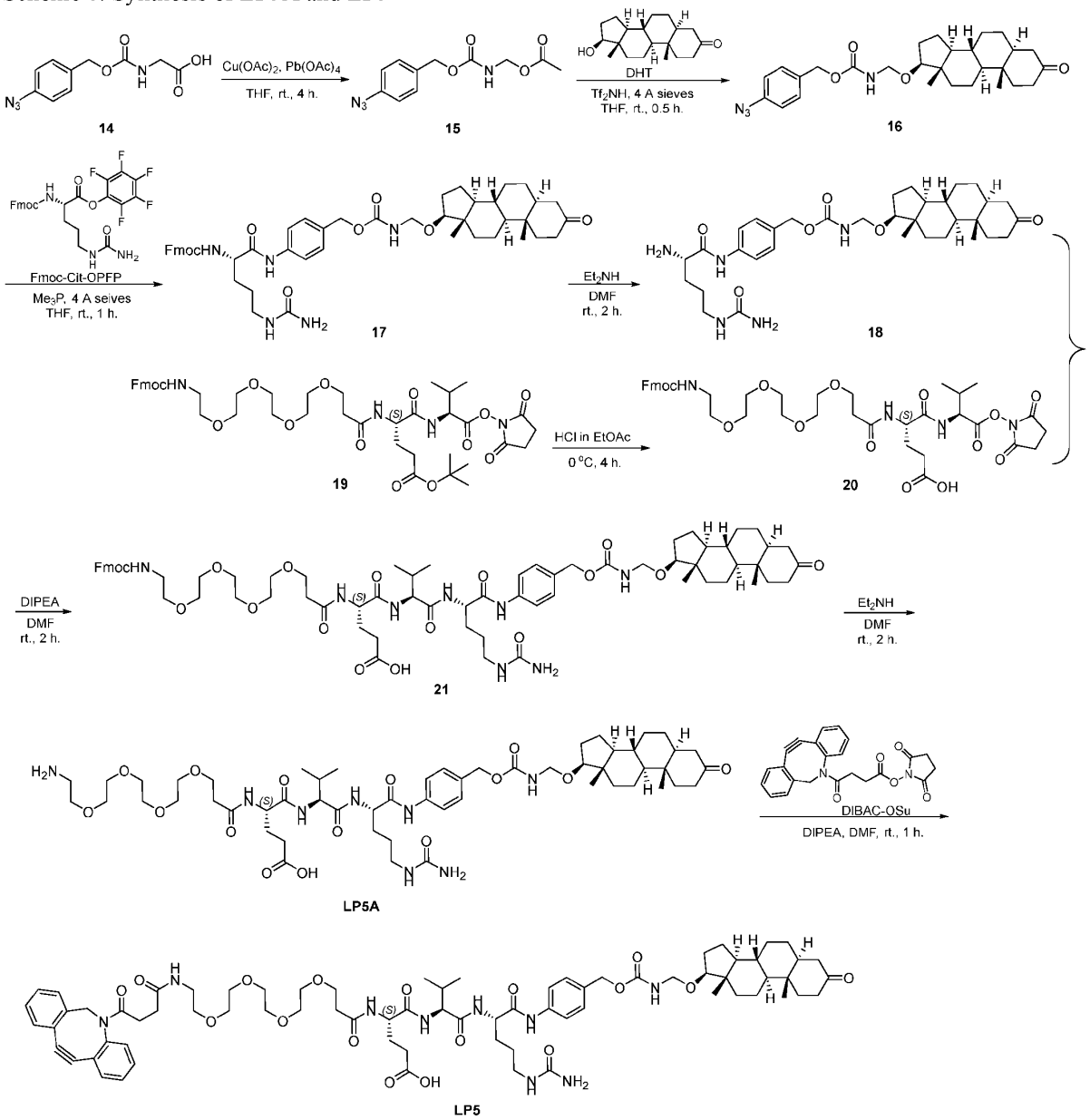
[0337] To a solution of compound **13** (50 mg, 44 μmol) in DMF (5 mL) were added **ProDHT** (17 mg, 44 μmol), DIPEA (11 mg, 88 μmol) and HOBt (3.0 mg, 22 μmol), and the reaction mixture was stirred at room temperature for 2 hours, which was monitored by LCMS. The resulting mixture was directly separated by reversed phase flash chromatography (0-100% acetonitrile in *aq.* TFA

(0.03%)) to give **LP4** (10 mg, 17% yield) as a red solid. ESI m/z: 1371 $(M + H)^+$, 1393 $(M + Na)^+$. ^1H NMR (400 MHz, $DMSO_{d6}$) δ 10.05 (s, 1H), 8.55 (t, J = 6.0 Hz, 1H), 8.40 (d, J = 8.8 Hz, 2H), 8.30 (t, J = 6.0 Hz, 1H), 8.20 (d, J = 6.0 Hz, 1H), 8.10 (d, J = 8.4 Hz, 1H), 7.75 (d, J = 8.8 Hz, 1H), 7.58 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H), 7.42 (t, J = 6.0 Hz, 1H), 7.29 (d, J = 8.4 Hz, 2H), 7.27 (br s, 1H), 6.03-6.00 (m, 1H), 5.52 (s, 2H), 4.97 (s, 2H), 4.60-4.58 (m, 1H), 4.55-4.45 (m, 3H), 4.20 (t, J = 6.0 Hz, 1H), 3.65-3.60 (m, 5H), 3.45-3.40 (m, 14H), 3.25-3.20 (m, 4H), 3.00 (s, 3H), 2.45-2.40 (m, 6H), 2.05-1.90 (m, 8H), 1.85-1.30 (m, 10H), 1.25-1.20 (m, 8H), 1.00 (s, 3H), 0.90-0.80 (m, 8H), 0.80 (s, 3H) ppm.

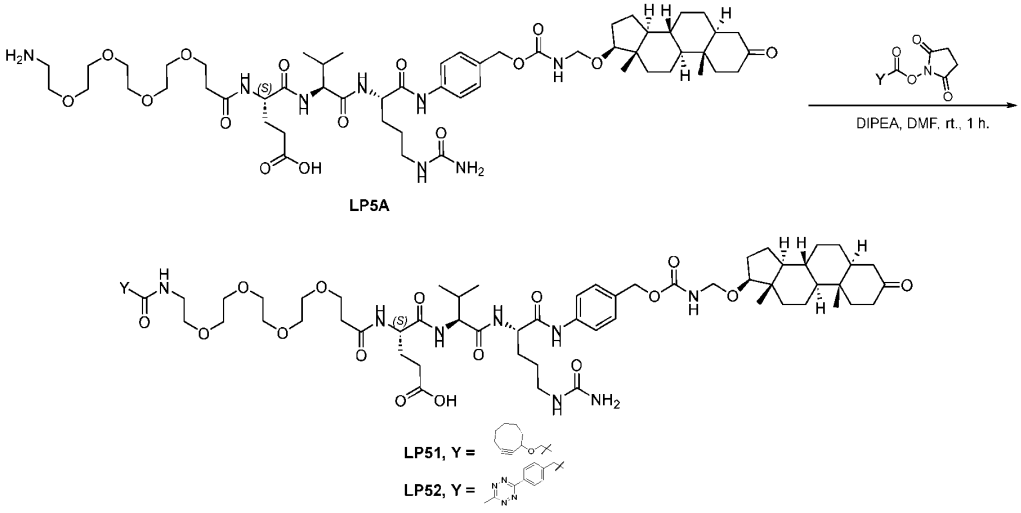
LP5A, LP5, LP51 and LP52

[0338] Synthesis of LP5 and LP5A is depicted in Scheme 6, below. Synthesis of LP51 and LP52 is depicted in Scheme 6a, below.

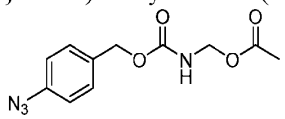
Scheme 6. Synthesis of LP5A and LP5



Scheme 6a. Synthesis of LP51 and LP52

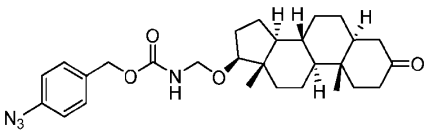


({[(4-azidophenyl)methoxy]carbonyl}amino)methyl acetate (15)



[0339] To a solution of compound **14** (CAS: 179806-96-3, synthesized according to *J. Chem. SOC., Perkin Trans. I,* **1996**, 1205-1211, 0.50 g, 2.0 mmol) in THF (15 mL) were added lead tetraacetate (2.7 g, 6.0 mmol) and copper acetate (35 mg, 0.20 mmol), and the reaction mixture was stirred at room temperature for 4 hours, which was monitored by LCMS. The resulting mixture was diluted with water (100 mL) and extracted with ethyl acetate (150 mL x 2). The combined organic solution was washed with brine (100 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by silica gel flash chromatography (0-15% ethyl acetate in petroleum ether) to give compound **15** (0.42 g, 80% yield) as a yellow solid. ESI m/z: 287.1 $(M + Na)^+$.

(4-azidophenyl)methyl *N*-({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamate (16)

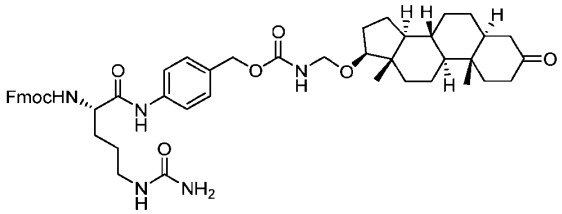


[0340] To a solution of compound **15** (0.18 g, 0.69 mmol) in dry THF (25 mL) were added 4 A molecular sieves and **DHT** (0.20 g, 0.69 mmol), and the mixture was stirred at room temperature

for half an hour before a solution of bis(trifluoromethane)sulfonimide (CAS: 82113-65-3, 0.27 g, 2.1 mmol) in dry THF (5 mL) was added into. The reaction mixture was stirred at room temperature for half an hour, which was monitored by LC and TLC. Both LC and TLC showed a new product formed and **DHT** no longer reduced (the rate of **DHT** vs. the new peak ~ 1 : 3 by LC). The reaction mixture was filtered and the solution was diluted with water (100 mL) and extracted with DCM (100 mL x 3). The combined organic solution was washed with brine (100 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by reversed phase flash chromatography (0-70% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give compound **16** (0.22 g, 65% yield) as a white solid and recycled **DHT** (20 mg, 10% recycled yield). ESI m/z: no mass signal.

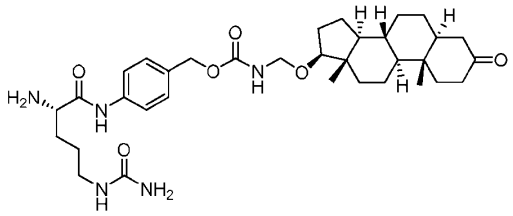
[0341] ^{1}H NMR (400 MHz, $DMSO_{d6}$) δ 7.36 (d, J = 4.4 Hz, 2H), 7.01 (d, J = 8.4 Hz, 2H), 5.11-5.08 (m, 2H), 4.75-4.64 (m, 2H), 2.44-2.25 (m, 3H), 2.12-1.98 (m, 3H), 1.85-1.79 (m, 2H), 1.74-1.66 (m, 1H), 1.59-1.50 (m, 3H), 1.45-1.21 (m, 8H), 1.15-1.05 (m, 1H), 1.03-1.00 (m, 3H), 0.96-0.84 (m, 2H), 0.77-0.74 (m, 3H) ppm.

(9*H*-fluoren-9-yl)methyl *N*-[(1*S*)-4-(carbamoylamino)-1-{[4-({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamate (17)



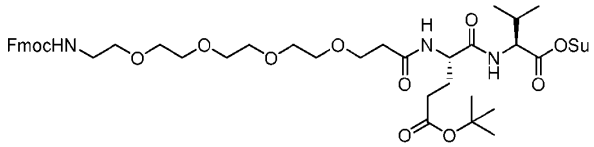
[0342] To a solution of compound **16** (0.20 g, 0.40 mmol) in dry THF (30 mL) were added 4 A molecular sieves (1.5 g) and Fmoc-Cit-OPFP (CAS: 1356537-03-5, 0.34 g, 0.60 mmol), and the mixture was at room temperature for half an hour. To the mixture was added trimethylphosphine (1 M in THF, 0.80 mL, 0.80 mmol) and the reaction mixture was stirred at room temperature for an hour until compound **16** was totally consumed, which was monitored by LCMS. The resulting mixture was filtered and the solution concentrated *in vacuo*. The residue was purified by reversed phase flash chromatography (0-70% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give compound **17** (50 mg, 15% yield) as a white solid. ESI m/z: 848.3 $(M + H)^+$ (very weak).

{4-[(2*S*)-2-amino-5-(carbamoylamino)pentanamido]phenyl}methyl *N*-({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamate (18)



[0343] To a solution of compound **17** (50 mg, 59 μmol) in DMF (2 mL) was added diethylamine (43 mg, 0.59 mmol), and the reaction mixture was stirred at room temperature for 2 hours until Fmoc was totally removed, which was monitored by LCMS. The resulting mixture was directly separated by reversed phase flash chromatography (0-70% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give compound **18** (25 mg, 68% yield) as a white solid. ESI m/z: 626.3 $(M + H)^+$.

tert-butyl (4*S*)-4-{[(2*S*)-1-[(2,5-dioxopyrrolidin-1-yl)oxy]-3-methyl-1-oxobutan-2-yl]carbamoyl}-4-[1-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-3,6,9,12-tetraoxapentadecan-15-amido]butanoate (19)

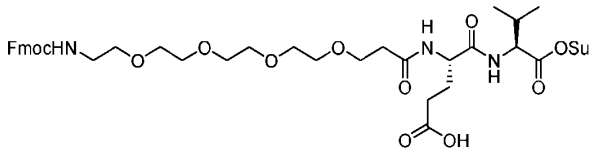


[0344] To a solution of H-Glu(O^{t}Bu)-Val-OH (29 g, 56 mmol) in DMF (200 mL) were added a solution of Fmoc-PEG$_4$-OSu (31 g, 53 mmol) in DCM (200 mL) and DIPEA (7.2 g, 56 mmol, 9.7 mL), and the reaction mixture was stirred at room temperature for 2 hours, which was monitored by LCMS. The volatiles was then removed *in vacuo*. The residue was diluted with water (100 mL), washed with MTBE (80 mL x 3) and acidified with citric acid to pH 5. The mixture was extracted with ethyl acetate (120 mL x 2) and the combined organic solution was washed with brine (60 mL x 2), dried over anhydrous sodium sulfate and concentrated *in vacuo* to give Fmoc-PEG$_4$-Glu(O^{t}Bu)-Val-OH (33 g) as yellow oil. ESI m/z: 772 $(M + H)^+$.

[0345] To a solution of HOSu (7.9 g, 68 mmol) in DMF (150 mL) and DCM (150 mL) were added DIC (6.5 g, 51 mmol) and Fmoc-PEG$_4$-Glu(O^{t}Bu)-Val-OH (33 g) obtained above. The reaction mixture was stirred at room temperature for 12 hours, which was monitored by TLC and LCMS.

The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue was diluted with water (200 mL) and extracted with ethyl acetate (150 mL x 3). The combined organic solution was washed with brine (150 mL x 3), dried over anhydrous sodium sulfate and concentrated *in vacuo* to give compound **19** (14 g) as yellow oil. ESI m/z 870 $(M + H)^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.64-7.58 (m, 2H), 7.42-7.37 (m, 2H), 7.34-7.28 (m, 2H), 4.86-4.76 (m, 1H), 4.56-4.34 (m, 3H), 4.28-4.18 (m, 1H), 3.76-3.73 (m, 3H), 3.66-3.56 (m, 13H), 3.39 (br d, J = 4.9 Hz, 1H), 2.81 (br s, 6H), 2.50-2.46 (m, 2H), 2.40-2.32 (m, 2H), 2.14-2.04 (m 1H), 1.97-1.81 (m, 4H), 1.44 (s, 9H), 1.05 (s, 3H), 1.04 (s, 3H) ppm.

(4*S*)-4-{[(2*S*)-1-[(2,5-dioxopyrrolidin-1-yl)oxy]-3-methyl-1-oxobutan-2-yl]carbamoyl}-4-[1-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-3,6,9,12-tetraoxapentadecan-15-amido]butanoic acid (20)

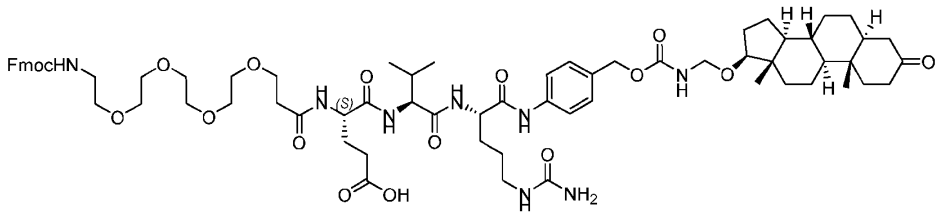


[0346] To a solution of compound **19** (Fmoc-PEG_4-Glu(O^tBu)-Val-OSu, 0.10 g, 0.12 mmol) in ethyl acetate (2 mL) was added a solution of HCl in ethyl acetate (4 N, 4 mL, 16 mmol), and the reaction mixture was stirred at 0 °C for 4 hours, which was monitored by LCMS. The volatiles were removed *in vacuo* and the residue was purified by reversed phase flash chromatography (0-50% acetonitrile in *aq.* formic acid (0.02%)) to give compound **20** (Fmoc-PEG4-Glu-Ala-OSu) (32 mg, 33% yield) as a white solid. ESI m/z: 813.2 $(M + H)^+$.

Alternative method (scale-up method):

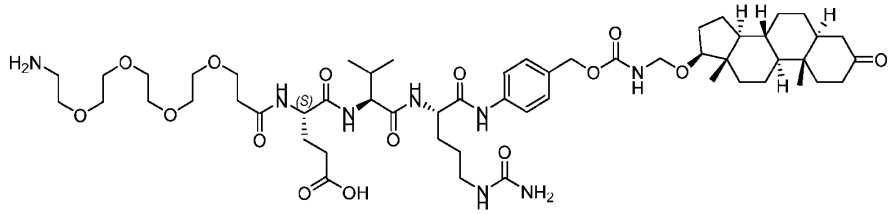
[0347] To a solution of compound **19** (0.14 g, 0.16 mmol, obtained above) in DCM (0.5 mL) was added TFA (0.72 g, 0.47 mL, 6.3 mmol), and the reaction mixture was stirred at room temperature for an hour, which was monitored by LCMS. The volatiles were removed *in vacuo* and the residue was triturated in MTBE (10 mL x 2). The off-white precipitates were collected by centrifuge to give compound **20** (0.13 g, 22% yield from H-Glu(O^tBu)-Val-OH) as an off-white solid. ESI m/z: 813 $(M + H)^+$.

(4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-[1-({[(9*H*-fluoren-9-yl)methoxy]carbonyl}amino)-3,6,9,12-tetraoxapentadecan-15-amido]butanoic acid (21)



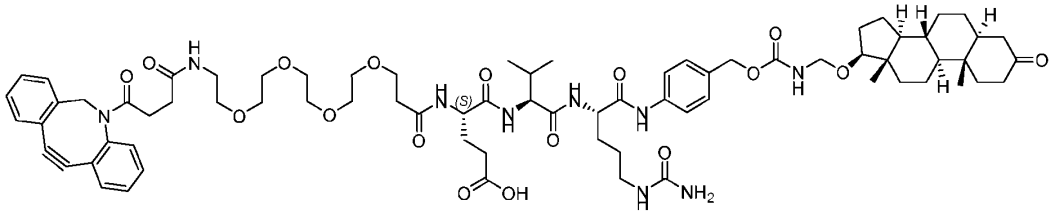
[0348] To a solution of compound **18** (25 mg, 40 μmol) in DMF (3 mL) were added DIPEA (16 mg, 0.12 mmol) and compound **20** (32 mg, 40 μmol), and the reaction mixture was stirred at room temperature for 2 hours, which was monitored by LCMS. The resulting mixture was purified by reversed phase flash chromatography (0-70% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give compound **21** (25 mg, 47% yield) as a white solid. ESI m/z: 698.3 $(M/2 + H)^{+}$.

(4*S*)-4-(1-amino-3,6,9,12-tetraoxapentadecan-15-amido)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}butanoic acid (LP5A)



[0349] To a solution of compound **21** (15 mg, 11 μmol) in DMF (2 mL) was added diethylamine (8.0 mg, 0.11 mmol), and the reaction mixture was stirred at room temperature for 2 hours until Fmoc was totally removed, which was monitored by LCMS. The resulting mixture was directly separated by prep-HPLC (10-95% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give **LP5A** (2.2 mg, 18% yield) as a white solid. ESI m/z: 1101 $(M + H)^{+}$.

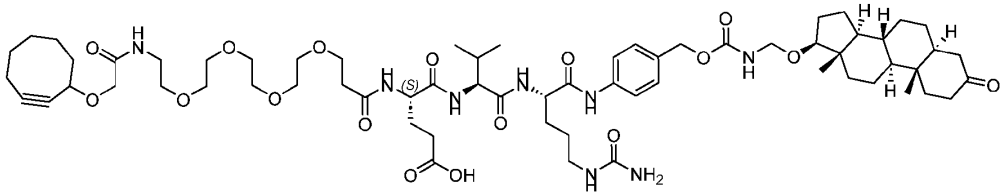
LP5: (4*S*)-4-[1-(4-{2-azatricyclo[10.4.0.0^{4,9}]hexadeca-1(12),4(9),5,7,13,15-hexaen-10-yn-2-yl}-4-oxobutanamido)-3,6,9,12-tetraoxapentadecan-15-amido]-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-{[4-({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}butanoic acid (LP5)



[0350] Following the similar procedure as **LP3A** to **LP3** except starting from **LP5A**, linker-payload **LP5** (2.7 mg, 18% yield) was obtained as a white solid. ESI m/z: 695.0 $(M/2 + H)^+$.

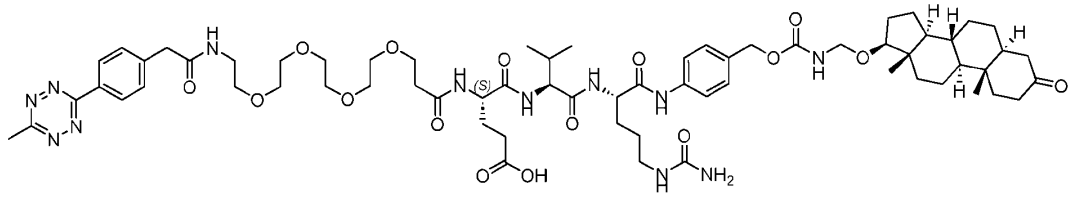
LP51 and LP52

(4S)-4-{[(1S)-1-{[(1S)-4-(carbamoylamino)-1-{[4-({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-{1-[2-(cyclooct-2-yn-1-yloxy)acetamido]-3,6,9,12-tetraoxapentadecan-15-amido}butanoic acid (LP51)



[0351] Following a similar procedure to **LP3A** to **LP3** except starting from **LP5A** reacting with COT-OSu, linker-payload **LP51** (1.0 mg, 16% yield) was obtained as a white solid. ESI m/z: 632.3 $(M/2 + H)^+$.

(4S)-4-{[(1S)-1-{[(1S)-4-(carbamoylamino)-1-{[4-({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]oxy}methyl)phenyl]carbamoyl}butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-(1-{2-[4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl]acetamido}-3,6,9,12-tetraoxapentadecan-15-amido)butanoic acid (LP52)

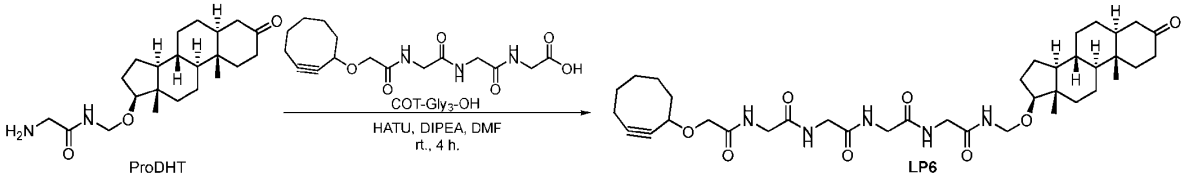


[0352] Following a similar procedure to **LP3A** to **LP3** except starting from **LP5A** reacting with MeTz-PhAc-NHS (CAS: 1644644-96-1), linker-payload **LP52** (0.9 mg, 14% yield) was obtained as a white solid. ESI m/z: 657.3 $(M/2 + H)^{+}$.

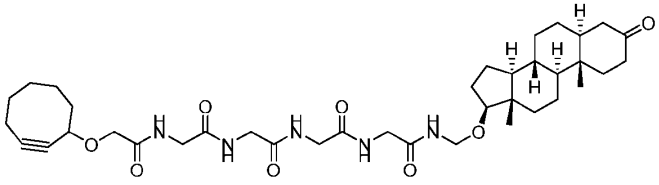
LP6

[0353] Synthesis of LP6 is depicted in Scheme 7, below.

Scheme 7. Synthesis of LP6. LP6 is disclosed as SEQ ID NO: 292.



2-(cyclooct-2-yn-1-yloxy)-N-{[({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)methyl]carbamoyl}methyl)carbamoyl]methyl}acetamide (LP6). LP6 is disclosed as SEQ ID NO: 292.



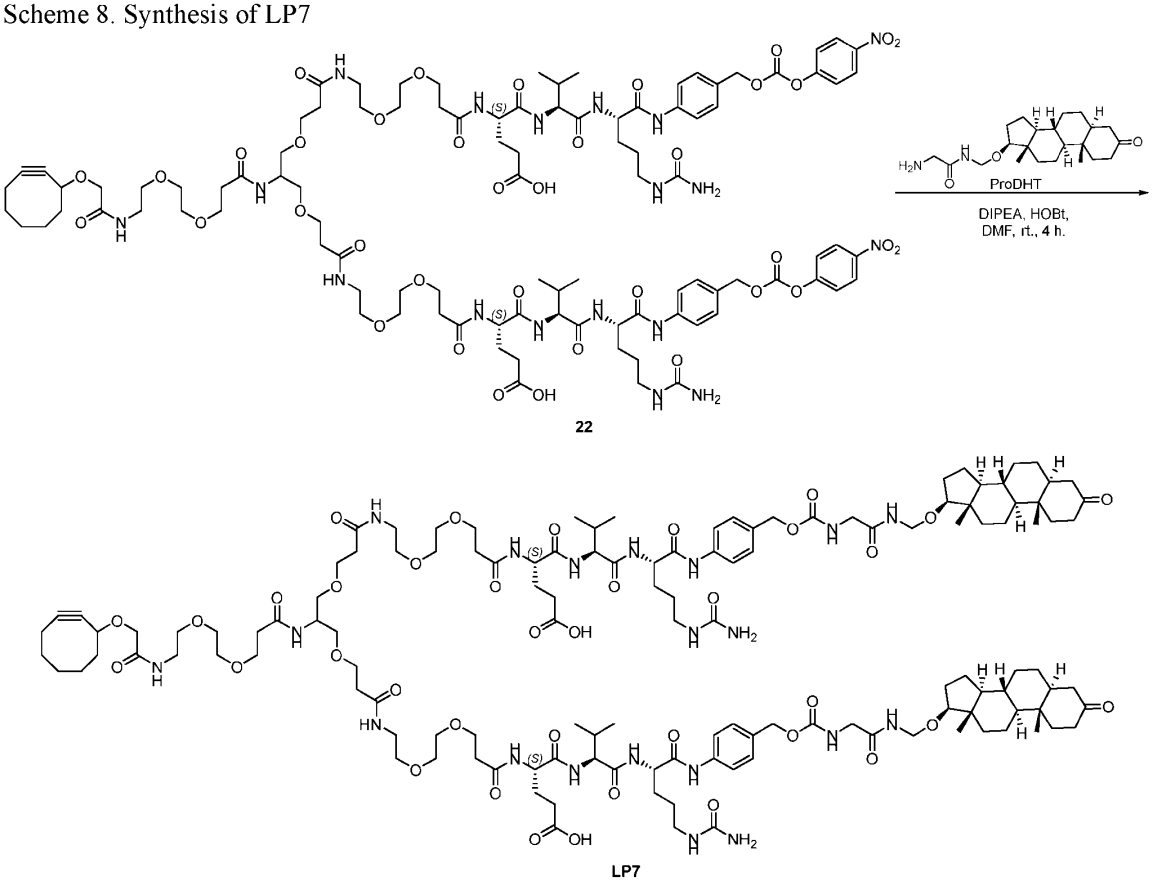
[0354] To a solution of COT-Gly$_3$-OH (CAS: 2504011-15-6, synthesized according to WO2022015656, 24 mg, 66 μmol) in DMF (3 mL) were added HATU (38 mg, 0.10 mmol) and DIPEA (26 mg, 0.20 mmol), and the mixture was stirred at room temperature for 10 minutes before

the addition of ProDHT (25 mg, 66 μmol). The reaction mixture was stirred at room temperature for 4 hours, which was monitored by LCMS. The resulting mixture was purified by prep-HPLC (5-95% acetonitrile in *aq.* TFA (0.01%)) to give **LP6** (7.2 mg, 15% yield) as a white solid. ESI m/z 422 ($M_{LP4} - M_{DHT} + H)^+$, 734 $(M + Na)^+$. ^1H NMR (400 MHz, $DMSO_{d6}$) δ 8.49 (t, J = 6.5 Hz, 1H), 8.20 (t, J = 5.7 Hz, 1H), 8.18-8.09 (m, 2H), 7.83 (t, J = 5.7 Hz, 1H), 4.65-4.51 (m, 1H), 4.51-4.49 (m, 1H), 4.35-4.29 (m, 1H), 4.00-3.66 (m, 10H), 2.50-2.05 (m, 8H), 1.98-1.60 (m, 8H), 1.66-1.08 (m, 15H), 0.97 (s, 3H), 0.90-0.83 (m, 2H), 0.68 (s, 3H) ppm.

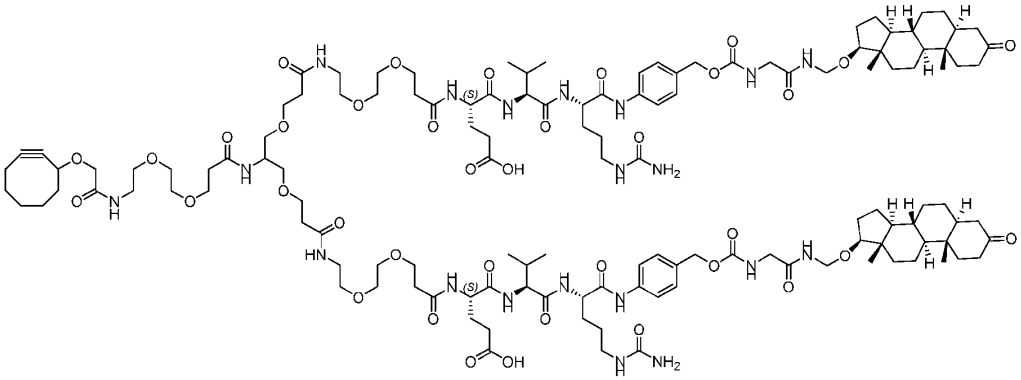
LP7

[0355] Synthesis of LP7 is depicted in Scheme 8, below.

Scheme 8. Synthesis of LP7



(4*S*)-4-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-[(4-{[({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}] heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)oxy]methyl}phenyl) carbamoyl]butyl]carbamoyl}-2-methylpropyl]carbamoyl}-4-(3-{2-[2-(3-{3-[2-({2-[2-(2-{[(1*S*)-1-{[(1*S*)-1-{[(1*S*)-4-(carbamoylamino)-1-[(4-{[({[({[(1*S*,2*S*,7*S*,10*R*,11*S*,14*S*,15*S*)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl] methyl}carbamoyl)oxy]methyl}phenyl)carbamoyl]butyl]carbamoyl}-2-methylpropyl] carbamoyl}-3-carboxypropyl]carbamoyl}ethoxy)ethoxy]ethyl} carbamoyl)ethoxy]-2-[3-(2-{2-[2-(cyclooct-2-yn-1-yloxy)acetamido]ethoxy}ethoxy)propanamido]propoxy} propanamido)ethoxy] ethoxy}propanamido)butanoic acid (LP7)

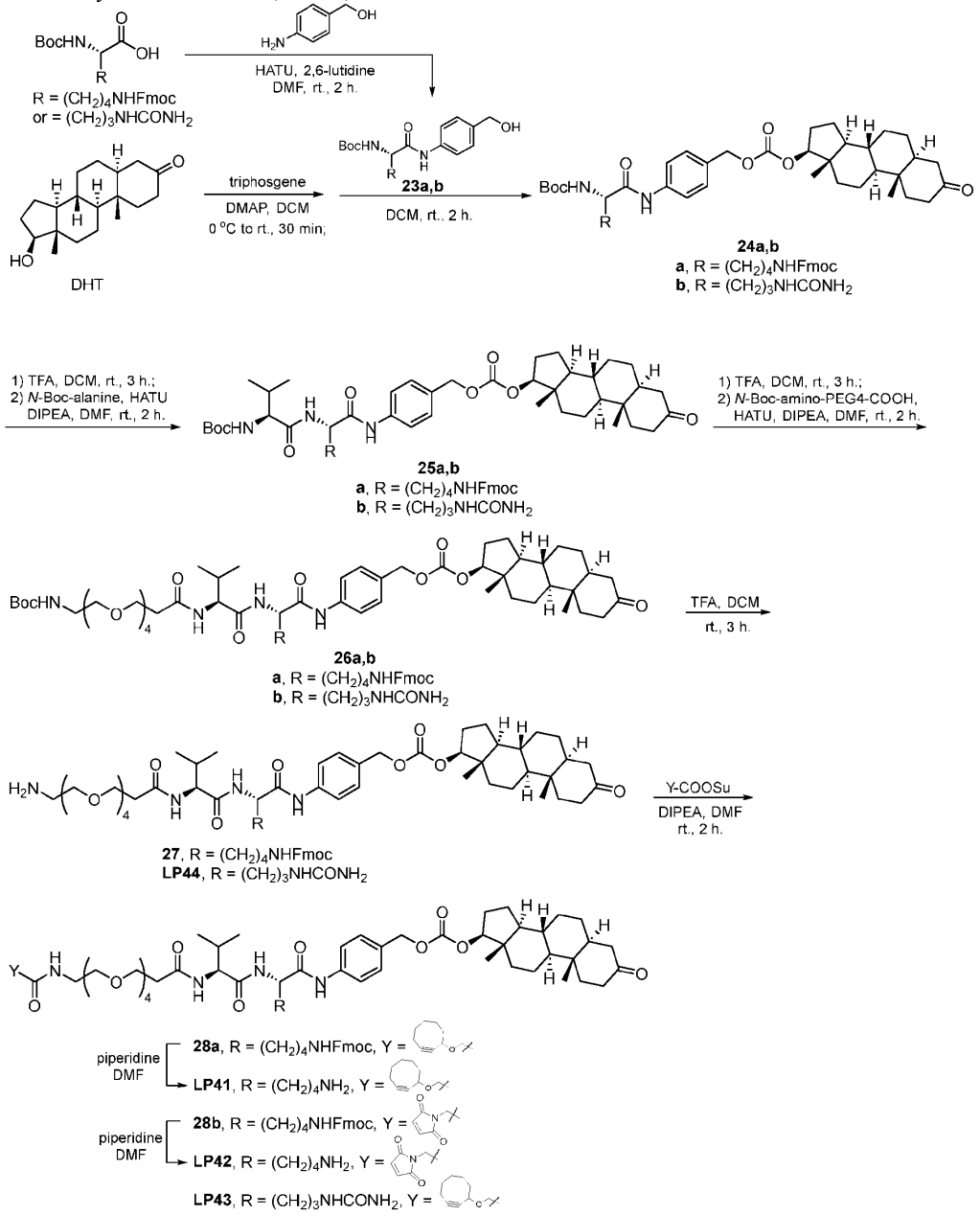


[0356] To a solution of compound **22** (0.18 g, 82 μmol) in DMF (5 mL) were added **ProDHT** (60 mg, 0.16 mmol) and HOBt (10 mg, 74 μmol), and the reaction mixture was stirred at room temperature for 4 hours, which was monitored by LCMS. The resulting mixture was directly separated by reversed phase flash chromatography (10-95% acetonitrile in *aq.* ammonium bicarbonate (10 mM)) to give **LP7** (20 mg, 9% yield) as a white solid. ESI m/z: 1331.7 $(M/2 + H)^{+}$. ^{1}H NMR (400 MHz, $DMSO_{d6}$) δ 10.0 (s, 2H), 8.55 (t, J = 6.4 Hz, 2H), 8.18 (d, J = 8.4 Hz, 2H), 8.08 (d, J = 8.4 Hz, 2H), 7.93 (t, J = 6.4 Hz, 2H), 7.75 (d, J = 7.2 Hz, 2H), 7.60-7.55 (m, 4H), 7.41 (t, J = 6.4 Hz, 2H), 7.29-7.27 (m, 4H), 6.00-5.98 (m, 2H), 5.40 (s, 4H), 5.00 (s, 4H), 4.60-4.50 (m, 2H), 4.45-4.35 (m, 2H), 4.30-4.20 (m, 5H), 4.20-4.15 (m, 2H), 3.80-3.75 (m, 1H), 3.75-3.50 (m, 11H), 3.55 (br, 10H), 3.50-3.45 (m, 6H), 3.45-3.40 (m, 10H), 3.10 (br, 6H), 3.00-2.90 (m, 3H), 2.25-2.15 (m, 16H), 1.80-1.60 (m, 19H), 1.50-1.45 (m, 20H), 1.45-1.40 (m, 20H), 0.95-0.90 (m, 16H), 0.85 (s, 6H), 0.60-0.55 (m, 9H) ppm.

LP41, LP42, LP43 and LP44

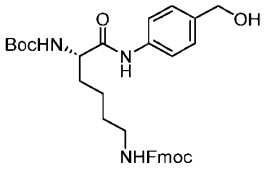
[0357] Synthesis of LP41, LP42, LP43 and LP44 is depicted in Scheme 9, below.

Scheme 9. Synthesis of LP41, LP42, LP43 and LP44



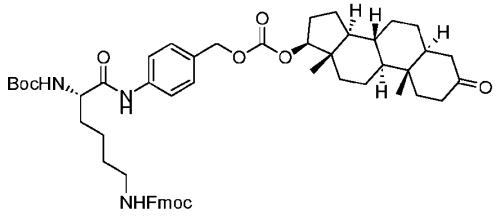
LP41, LP42, LP43 and LP44

tert-butyl N-[(1S)-5-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)-1-{[4-(hydroxymethyl)phenyl]carbamoyl}pentyl]carbamate (23a)



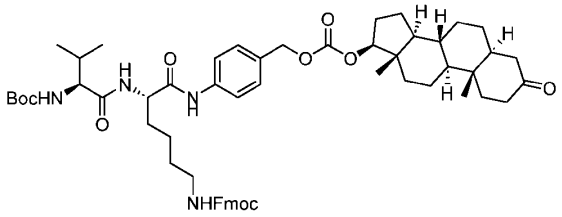
[0358] To a solution of Boc-Lys(Fmoc)-OH (1.0 g, 2.1 mmol) in DMF(10 mL) were added (4-aminophenyl)methanol (0.26 g, 2.1 mmol), HATU (1.2 g, 3.2 mmol) and 2,6-Lutidine (0.68 g, 6.3 mmol), and the mixture was stirred at room temperature for 3 hours. The resulting mixture was purified by reversed phase flash chromatography (0-95% acetonitrile in *aq.* TFA (0.1%)) to give compound **23a** (1.0 g, 82% yield) as a white solid. ESI m/z: 574.3 $(M + H)^+$.

{4-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-6-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)hexanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl carbonate (24a)



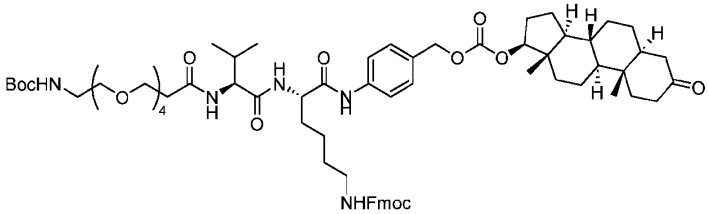
[0359] To a solution of **DHT** (0.50 g, 1.7 mmol) in DCM (10 mL) was added DMAP (1.2 g, 10 mmol). After the mixture was cooled to 0 °C, triphosgene (0.15 g, 0.85 mmol) was added into the mixture. The reaction mixture was stirred at room temperature for an hour, and to the mixture was added **23a** (1.0 g, 3.4 mmol). The mixture was stirred at room temperature for 2 hours. The resulting mixture was concentrated *in vacuo* and the residue was purified by flash chromatography (5-95% acetonitrile in *aq.* TFA (0.1%)) to give **24a** (0.50 g, 50% yield) as a white solid. ESI m/z: 890.2 $(M + H)^+$.

{4-[(2S)-2-[(2S)-2-{[(tert-butoxy)carbonyl]amino}-3-methylbutanamido]-6-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)hexanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadecan-14-yl carbonate (25a)



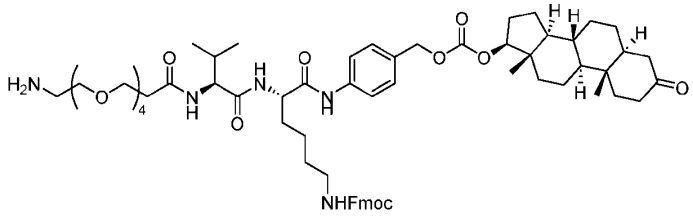
[0360] To a solution of compound **24a** (0.50 g, 1.7 mmol) in DCM (8 mL) was added TFA (1.0 mL), and the reaction mixture was stirred at room temperature for 3 hours. The resulting mixture was concentrated *in vacuo*, and the residue was dissolved in DMF (5 mL). To the solution were added Boc-valine (0.11 g, 0.5 mmol), HATU (0.29 g, 0.75 mmol) and DIPEA (0.19 g, 1.5 mmol), and the reaction mixture was stirred at room temperature for 3 hours. The resulting mixture was purified by reversed phase flash chromatography (0-95% acetonitrile in *aq.* TFA (0.1%)) to give compound **25a** (0.30 g, 54% yield) as a white solid. ESI m/z: 990.2 $(M + H)^+$.

{4-[(2S)-2-[(2S)-2-(1-{[(tert-butoxy)carbonyl]amino}-3,6,9,12-tetraoxapentadecan-15-amido)-3-methylbutanamido]-6-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)hexanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadecan-14-yl carbonate (26a)



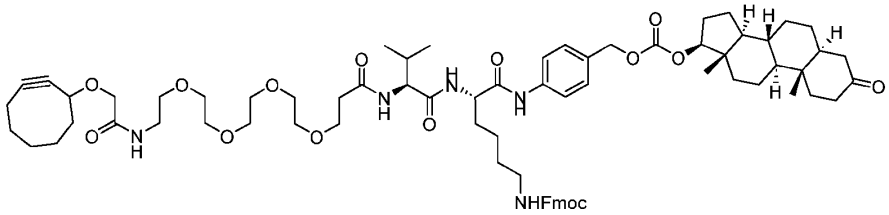
[0361] Following the similar procedure as **25a** except using *N*-Boc-PEG4-acid instead of Boc-valine, compound **26a** (0.20 g, 58% yield) as a white solid. ESI m/z: 1237.6 $(M + H)^+$.

{4-[(2S)-2-[(2S)-2-(1-amino-3,6,9,12-tetraoxapentadecan-15-amido)-3-methylbutanamido]-6-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)hexanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl carbonate (27)



[0362] To a solution of **26a** (0.20 g, 0.16 mmol) in DCM (8 mL) was added TFA (1.0 mL), and the mixture was stirred at room temperature for 3 hours. The resulting mixture was concentrated *in vacuo* and the residue was purified by reversed phase flash chromatography (5-95% acetonitrile in *aq.* TFA (0.1%)) to give compound **27** (0.14 g, 77% yield) as a white solid. ESI m/z: 1137.5 $(M + H)^{+}$.

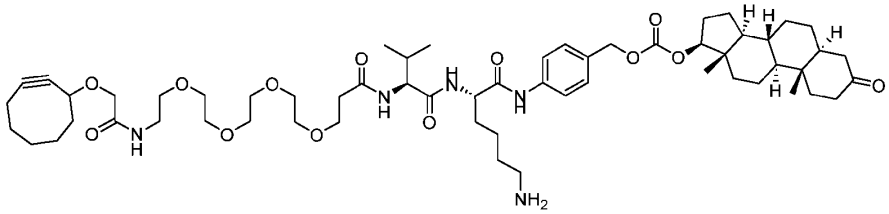
{4-[(2S)-2-[(2S)-2-{1-[2-(cyclooct-2-yn-1-yloxy)acetamido]-3,6,9,12-tetraoxapentadecan-15-amido}-3-methylbutanamido]-6-({[(9H-fluoren-9-yl)methoxy]carbonyl}amino)hexanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl carbonate (28a)



[0363] To a solution of **27** (0.14 g, 0.12 mmol) in DMF (2 mL) were added COT-OSu (34 mg, 0.12 mmol) and DIPEA (46 mg, 0.36 mmol), and the reaction mixture was stirred at room temperature for 3 hours. The resulting mixture was directly separated by reversed phase flash chromatography (5-95% acetonitrile in *aq.* TFA (0.1%)) to give **28a** (90 mg, 57% yield) as a white solid. ESI m/z: 1301.7 $(M + H)^{-}$.

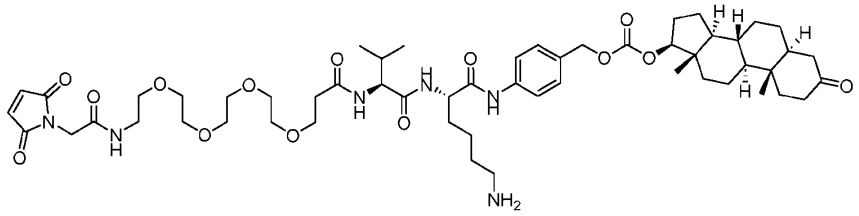
[0364] {4-[(2S)-6-amino-2-[(2S)-2-{1-[2-(cyclooct-2-yn-1-yloxy)acetamido]-3,6,9,12-tetraoxapentadecan-15-amido}-3-methylbutanamido]hexanamido]phenyl}methyl

(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl carbonate (LP41)



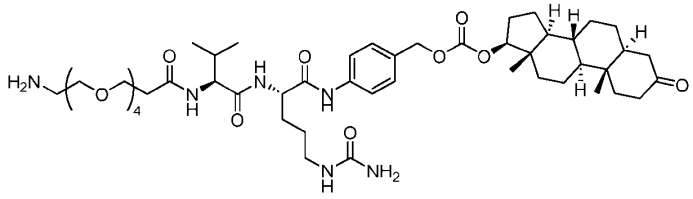
[0365] To a solution of **28a** (90 mg, 68 μmol) in DMF (2 mL) was added piperidine (0.2 mL), and the reaction mixture was stirred at room temperature for 3 hours. The mixture was directly separated by reversed phase flash chromatography (5-95% acetonitrile in *aq.* TFA (0.1%)) to give **LP41** (40 mg, 54% yield) as a white solid. ESI m/z: 1079.5 $(M + H)^{+}$.

{4-[(2S)-6-amino-2-[(2S)-2-{1-[2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido]-3,6,9,12-tetraoxapentadecan-15-amido}-3-methylbutanamido]hexanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl carbonate (LP42)



[0366] Following the similar procedure as **LP41** except using AMAS (CAS: 55750-61-3) instead of COT-OSu, linker-payload **LP42** (4.5 mg, 20% yield) as a white solid. ESI m/z: 1051.6 $(M + H)^{+}$.

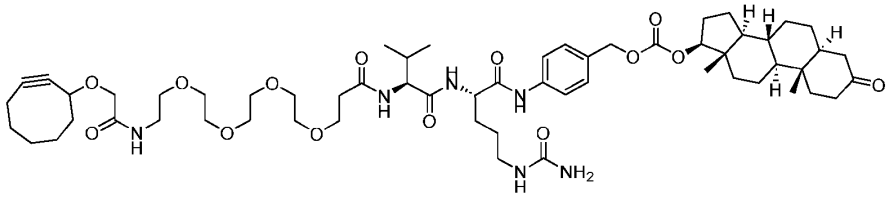
{4-[(2S)-2-[(2S)-2-(1-amino-3,6,9,12-tetraoxapentadecan-15-amido)-3-methylbutanamido]-5-(carbamoylamino)pentanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl carbonate (LP44)



[0367] Following the similar procedures as compound **27** except starting from *N*-Boc-Cit-OH, compound **23b**, **24b**, **25b**, **26b** and **LP44** were obtained.

LP44 (20 mg, 15% total yield): ESI m/z: 943.5 $(M + H)^+$.

{4-[(2S)-5-(carbamoylamino)-2-[(2S)-2-{1-[2-(cyclooct-2-yn-1-yloxy)acetamido]-3,6,9,12-tetraoxapentadecan-15-amido}-3-methylbutanamido]pentanamido]phenyl}methyl (1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl carbonate (LP43)

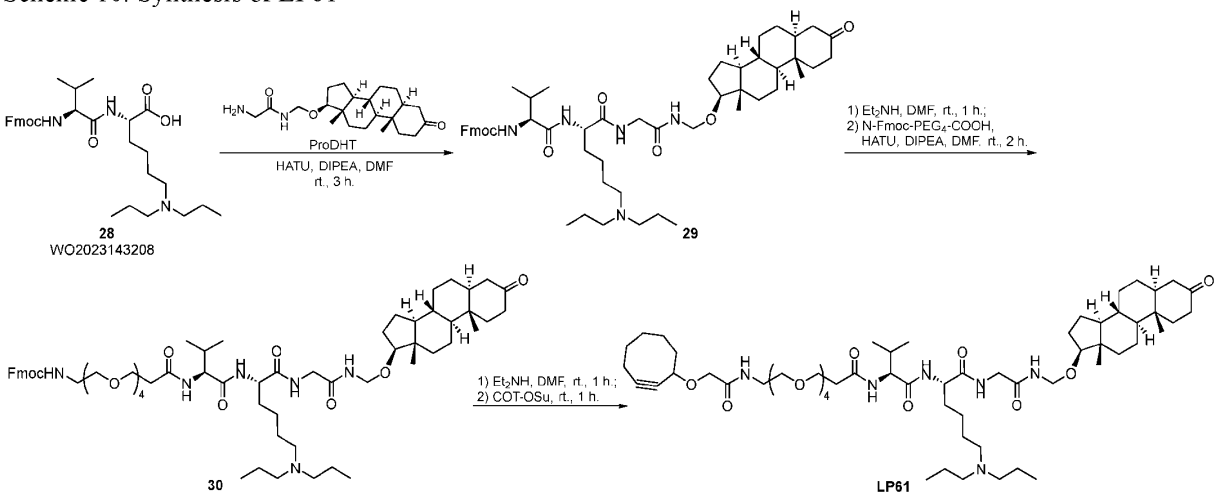


[0368] Following the similar procedure as **LP41** except using **LP44** instead of compound **27**, linker-payload **LP43** (2.5 mg, 60% yield) was obtained as a white solid. ESI m/z: 1107.7 $(M + H)^+$.

LP61 and LP61A

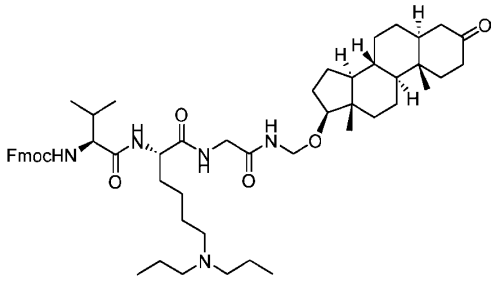
[0369] Synthesis of LP61 is depicted in Scheme 10, below.

Scheme 10. Synthesis of LP61



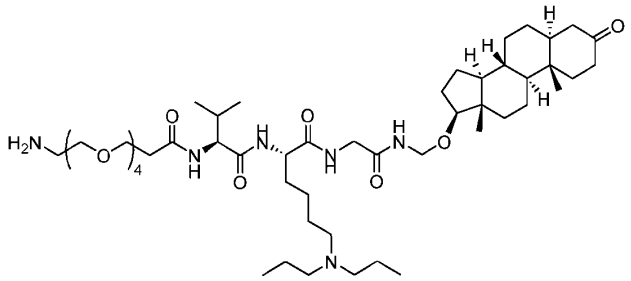
LP61 and LP61A

(9H-fluoren-9-yl)methyl N-[(1S)-1-{[(1S)-1-({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)-5-(dipropylamino)pentyl]carbamoyl}-2-methylpropyl]carbamate (29)



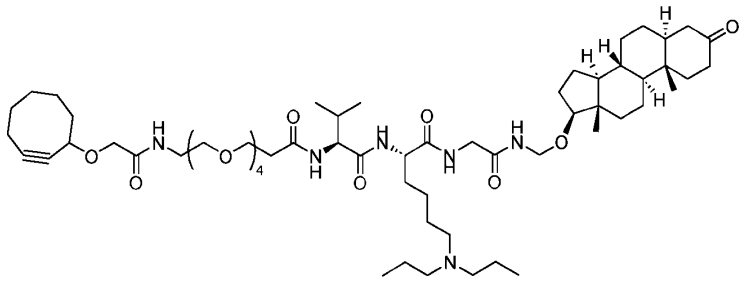
[0370] To a solution of compound **28** (synthesized according to WO2023143208, 33 mg, 60 μmol) in DMF (3 mL) were added HATU (38 mg, 0.10 mmol) and DIPEA (26 mg, 0.20 mmol), and the mixture was stirred at room temperature for 10 minutes before the addition of ProDHT (19 mg, 50 μmol). The reaction mixture was stirred at room temperature for 4 hours, which was monitored by LCMS. The resulting mixture was purified by prep-HPLC (5-95% acetonitrile in *aq.* TFA (0.01%)) to give compound **29** (10 mg, 22% yield) as a white solid. ESI m/z 910.6 $(M + H)^{+}$.

1-amino-N-[(1S)-1-{[(1S)-1-({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)-5-(dipropylamino)pentyl]carbamoyl}-2-methylpropyl]-3,6,9,12-tetraoxapentadecan-15-amide (LP61A)



[0371] Following the similar procedures as **LP2A** except using compound **29** instead of Fmoc-vcPAB-ProDHT, linker-payload **LP61A** (6.0 mg, 58% yield) was obtained as a white solid. ESI m/z: 935.7 $(M + H)^{+}$.

1-[2-(cyclooct-2-yn-1-yloxy)acetamido]-N-[(1S)-1-{[(1S)-1-({[({[(1S,2S,7S,10R,11S,14S,15S)-2,15-dimethyl-5-oxotetracyclo[8.7.0.0^{2},^{7}.0^{11},^{15}]heptadecan-14-yl]oxy}methyl)carbamoyl]methyl}carbamoyl)-5-(dipropylamino)pentyl]carbamoyl}-2-methylpropyl]-3,6,9,12-tetraoxapentadecan-15-amide (LP61)



[0372] Following the similar procedure as **28a** except using **LP61A** instead of compound **27**, linker-payload **LP61** (0.5 mg, 21% yield) was obtained as a white solid. ESI m/z: 1100.0 $(M + H)^{+}$.

[0373]

Example 9: Preparation of Antibody-Drug Conjugates (ADCs)

[0374] Generic procedures for making site-specific conjugates are disclosed below and shown in **Figure 8**.

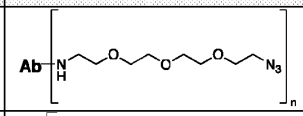
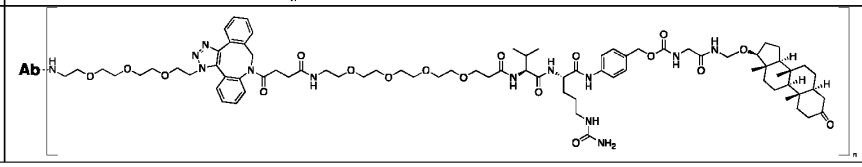
[0375] Aglycosylated human antibody IgG (IgG1, IgG4, etc.) containing an N297Q or N297D mutation were used in ADC conjugations. Conjugation was conducted via a two-step process (**Figure 8A**), and the conjugation results with the structures and MS-DAR values are summarized in Table 5.

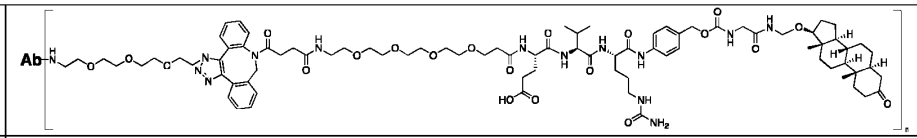
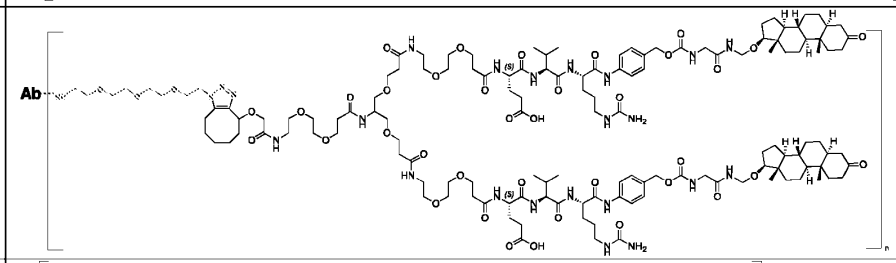
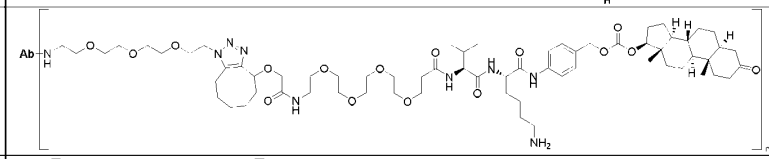
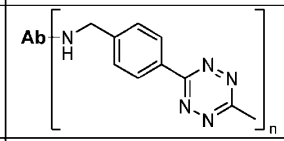
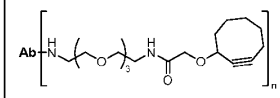
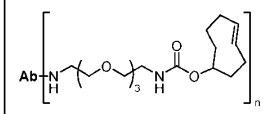
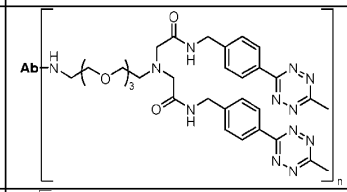
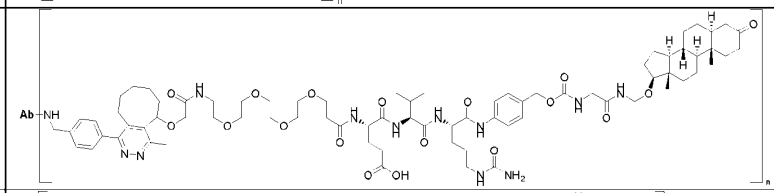
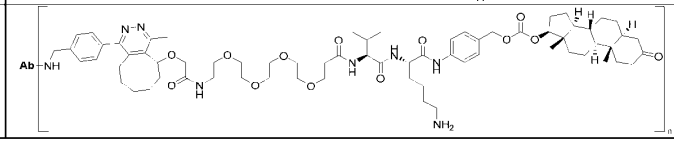
Table 5. List of Antibodies, Azido functionalized Antibodies, and ADCs

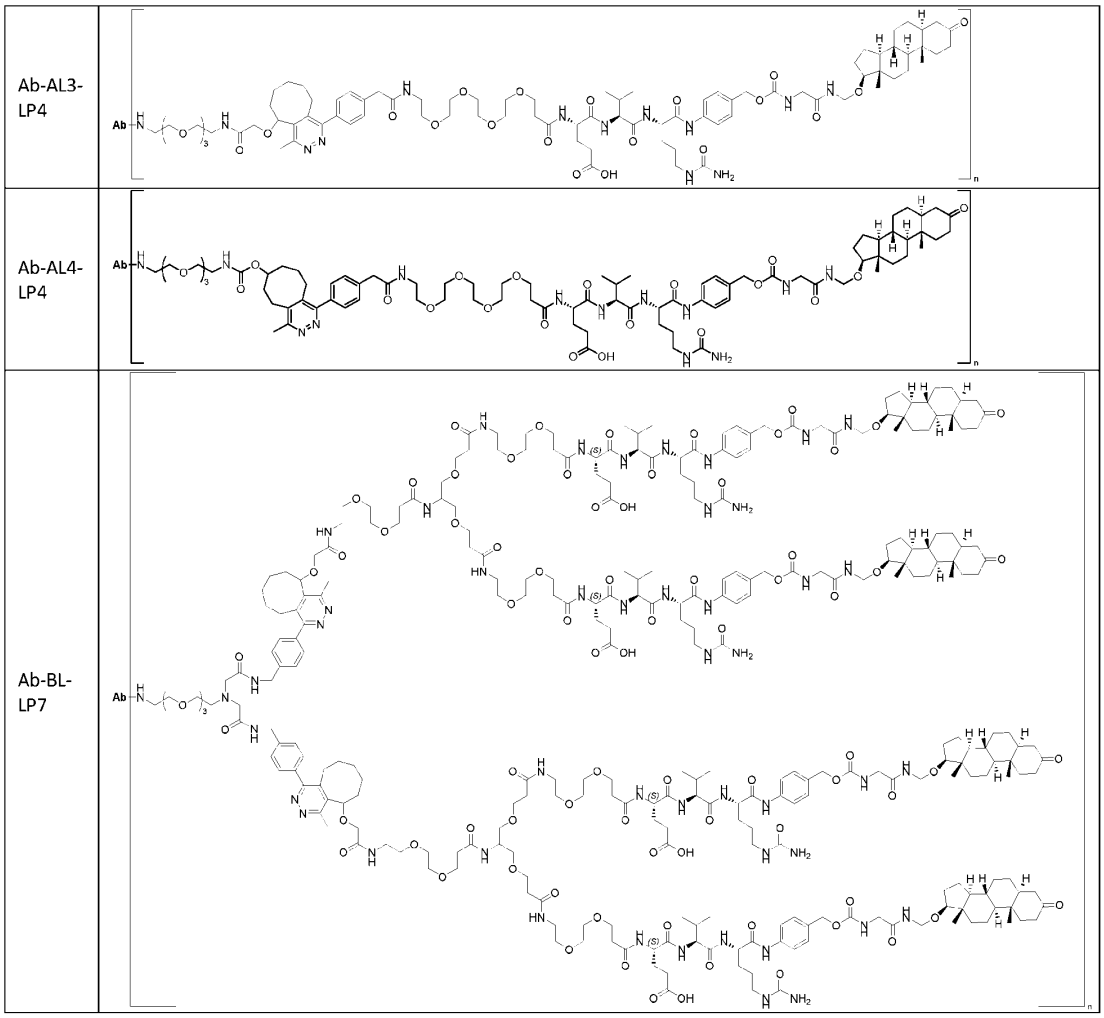
ADC description	Linker-payload	DAR	ES-MS
aCACNG mAb1			145580
aCACNG mAb2			144909
aCACNG mAb3			144426
aCACNG mAb4			145183
aCACNG mAb5			144332
aCACNG mAb6			145091
aCACNG mAb7			145075
Fel D1 mAb			145439

aCACNG1 mAb-M404	Ab1-$(AL)_4$	4.0	146389
aCACNG1 mAb-M404	Ab2-$(AL)_4$	3.9	145713
aCACNG1 mAb-M404	Ab3-$(AL)_4$	4.0	145227
aCACNG1 mAb-M404	Ab4-$(AL)_4$	4.0	145985
aCACNG1 mAb-M404	Ab5-$(AL)_4$	4.0	145135
aCACNG1 mAb-M404	Ab6-$(AL)_4$	4.0	145894
aCACNG1 mAb-M404	Ab7-$(AL)_4$	4.0	145874
aCACNG1 mAb-M404	Cont.Ab-$(AL)_4$	4.0	146248
ADC1	Ab1-$(AL\text{-}LP)_4$	4.0	151671
ADC2	Ab2-$(AL\text{-}LP)_4$	3.9	150977
ADC3	Ab3-$(AL\text{-}LP)_4$	3.9	150486
ADC4	Ab4-$(AL\text{-}LP)_4$	3.9	151244
ADC5	Ab5-$(AL\text{-}LP)_4$	4.0	150394
ADC6	Ab6-$(AL\text{-}LP)_4$	4.0	151153
ADC7	Ab7-$(AL\text{-}LP)_4$	4.0	151133
ADC9	Ab-$(AL\text{-}LP)_4$	3.9	151489
ADC10	Ab2-$(AL\text{-}LP)_4$	3.8	151756
ADC11	Ab7-$(AL\text{-}LP)_4$	3.7	151656
Control. ADC1	Control.Ab-$(AL\text{-}LP)_4$	4.0	151543
Control. ADC2	Control. Ab-$(AL\text{-}LP)_4$	3.8	152038

Table 6: Select exemplified antibody-linkers and ADCs according to the present disclosure

ADC #	ADC structure
Ab-Handle = Ab-AL	
Ab-AL-LP2	

Ab-AL-LP3	
Ab-AL-LP7	
Ab-AL-LP41	
Ab-Handle 2 = Ab-AL2	
Ab-Handle 3 = Ab-AL3	
Ab-Handle 4 = Ab-AL4	
Ab-Handle 4 = Ab-BL	
Ab-AL2-LP32	
Ab-AL2-LP41	



[0376] Step 1: site-specific conjugation of Handle-functionalized amine with an Antibody generated a modified antibody conjugate containing 1-4 handles per antibody.

[0377] Aglycosylated human antibody IgG containing an N297Q mutation or N297D mutation in BupH buffer (pH7.4) was mixed with >=100 molar equivalents of non-branched or branched Handle-amine (**AL**). The resulting solution was mixed with transglutaminase (350 U/mL; 1U mTG per mg of antibody, SLCK1576, Sigma; or 25 U/mL; 1U mTG per mg of antibody, Zedira, Darmstadt, Germany; or 10 U/mL; 0.06 mg mTG per mg of antibody, Modernist Pantry-ACTIVA TI contains Maltodextrin from Ajinomoto, Japan) resulting in a final concentration of the antibody

at 0.5-20mg/mL. The reaction mixture was incubated at 25-37°C for 24 hours while gently shaking while monitored by ESI-MS. Upon the completion, the excess amine and mTG were removed by size exclusion chromatography (SEC) or protein A column chromatography. The conjugate was characterized by UV-Vis, SEC and ESI-MS.

[0378] Step 2: click reactions between Handle-functionalized antibodies (Ab-Handle) and a Linker-Payload (LP) in Table 3 to generate the site-specific ADCs.

[0379] The Handle-functionalized antibody (Ab-(**AL**)$_n$, 1-20 mg/mL) in PBS (pH 7.4) was incubated with ≥ 2-10 molar equivalents of a linker-payload (LP) dissolved in an organic solvent such as DMSO or DMA (10 mg/mL) to have the overall reaction mixture containing 5-15% organic solvent (v/v), at 25-37 °C for 1-48 hours while gently shaking. The reaction was monitored by ESI-MS. Upon completion, the excess amount of LP and organic solvent were removed via a desalting column with BupH (pH 7.4), and protein aggregates (if any) were removed by size exclusion chromatography (SEC). The purified conjugate, Ab-(**AL**-LP)$_n$ ADC or Ab-(**AL**-(LP)$_2$)$_4$ ADC, was concentrated, sterile filtered, and characterized by UV-Vis, SEC, and ESI-MS. Conjugate monomer purity was > 95% by SEC.

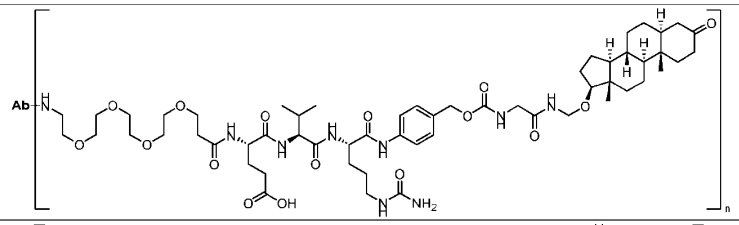
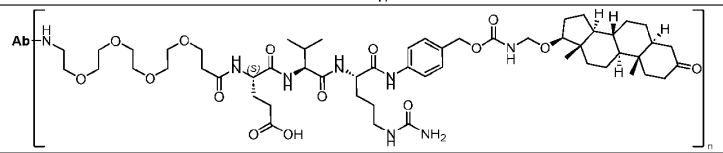
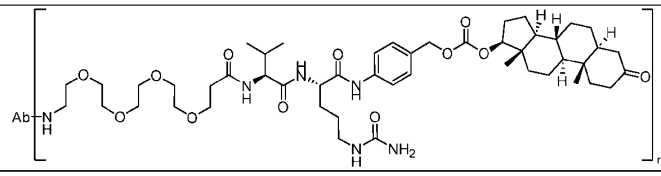
[0380] All ADCs were purified by SEC using an ÄKTA instrument from Cytiva, using a 16/600 Superdex® 200 column, eluting with DPBS, at a flow rate of 1.5 mL/min at pH 7.4. The DAR values of the ADCs were measured by ESI-MS. A mass increase of 4 x **LP** from **Ab-[AL]$_4$** was observed, correlating to 4DAR ADC.

Detailed conjugation procedure

[0381] A representative 4DAR ADC from Approach I is exemplified following. The aglycosylated anti-CACNG1 human IgG antibody containing an N297Q mutation was mixed with 100 molar equivalents of an azido-dPEG3-amine (**AL1**, MW 218.26 g/mol). The resulting solution was mixed with microbial transglutaminase (350U/mL; 1U mTG per mg of antibody, Sigma) resulting in a final concentration of the antibody at 3.4 mg/mL. The reaction mixture was incubated at 32 °C for one hour while gently shaking while monitored by ESI-MS. Upon the completion, the excess amine and mTG were removed by size exclusion chromatography (SEC). The conjugate was characterized by UV-Vis, SEC and ESI-MS. The azido linkers attached antibody resulted in a 811Da mass increase compared to mAb, indicating 4 AL was conjugated to the antibody (Ab-(AL)$_4$) with 4 azido handles.

[0382] The site-specific antibody azido conjugate (6.9 mg/mL) in PBS (pH7.0) was mixed with 6 molar equivalents of linker-payload (**LP1**) in 2mM of DMSO to have the reaction mixture containing 12% organic solvent (v/v), and the solution was set at 37°C for 4 hours while gently shaking. The reaction was monitored by ESI-MS. Upon completion, the excess amount of linker-payload and protein aggregates were removed by size exclusion chromatography (SEC). The purified conjugate was concentrated, sterile filtered and characterized by UV-Vis, SEC and ESI-MS. Conjugates monomer purity was 99.8% by SEC. The drug attached antibody resulting in a 5264 Da mass increase for the DAR4 conjugate. Conjugates monomer purity was >99% by SEC.

Table 7: Select ADCs prepared according to the one-step procedure as shown in **Fig. 8B**

LP #	ADC structure
Ab-LP3A	
Ab-LP5A	
Ab-LP44	

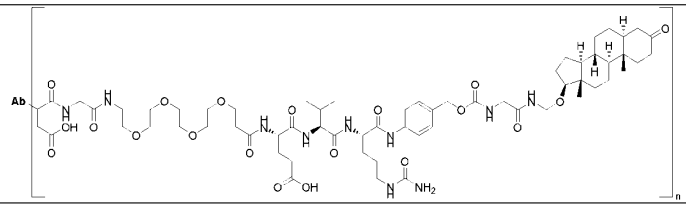
Interchain Disulfide Conjugation

[0383] The antibodies (2 mg/ml) in 50 mM HEPES, 2 mM EDTA, pH 7.4, were treated with 1 mM dithiothreitol or 5 mM TCEP at 37 ◦C for 90 min. After Amicon filtration (30K) the maleimido linker payload derivative (1.0-2.0 equivalents/SH group of the cysteine residue, LP33 or LP42) in DMSO or DMA (8 mM solution) was added to the reduced antibody. After 1 h the completion of the reaction was confirmed by LC-MS monitoring. The conjugates were purified by size exclusion chromatography under isocratic buffer elution condition using PBS (1X) pH 7.4 or Hydrophobic interaction Chromatography under gradient elution condition using 3M NaCl in 50 mM Sodium

Phosphate as binding buffer at pH 7.4 and 50 mM Sodium phosphate with 15% - 20% IPA as elution buffer at pH 7.4. Pure fractions were concentrated, and buffer exchanged into PBS (1 X) pH 7.4 by 30 K Amicon filter followed by formulation with 5% (v/v) glycerol and sterile filtered. Protein and linker payload concentrations were determined by UV spectral analysis. Size-exclusion HPLC established that all conjugates used were >95% monomeric, and RP-HPLC established that there was <0.5% unconjugated linker payload.

[0384] It is known in the art that the 5-membered succinimide ring formed during the conjugation exists as an equilibrium between the closed ring and two open-ring isoforms by hydrolysis under neutral or alkaline conditions.

Table 8: Select ADC prepared according to the Cys-maleimide procedure as shown in **Fig. 8C**

LP #	ADC structure
Ab-LP33	

Example 10: Characterization of Exemplified ADCs

[0385] Select ESI-MS spectra of ADCs and Ab-Handles are shown in **Figures 9-11**.

Example 10A: SDS-PAGE for analysis of ADC integrity and purity

[0386] In one method, SDS-PAGE running conditions include non-reduced and reduced samples (1-2µg) along with Precision Plus Protein Dual Color Standards (Bio-rad, 500 µl, Cat# 1610374) are loaded per lane in (1.0 mm × 10 well) Novex 4-20% No Tris-Glycine Gel and is run at 180V, 300mA, for 80 minutes. A non-reduced sample is prepared using NuPAGE® LDS Sample Buffer (4X) (Thermo Fisher Scientific, Cat#1887691) and the reduced sample is prepared with SDS sample buffer (4X) containing 10% sample reducing agent (10X) (Thermo Fisher Scientific, Cat#1769410).

[0387] Molecular weights of the antibodies and ADCs on SDS-PAGE are determined under non-reducing and reducing conditions. The mass shifts may not be obvious under non-reducing conditions due to relatively small percentages of mass changes. However, the masses of the heavy

chains are increased from the naked antibodies to the azido-functionalized antibodies, and further to the ADC conjugates.

Example 10B: Size Exclusion Chromatography (SEC) for ADC analysis and purification

[0388] To determine the purity of antibody drug conjugates, size exclusion chromatography is performed. Analytical SEC experiments are run using a Thermo UltiMate™ 3000 instrument, on a XBridge Protein BEH SEC Column (Waters, 200Å, 3.5 µm, 7.8 mm X 300 mm), and each sample (30-40µg, 20µL) are run at flow rate of 0.5mL/min using PBS pH 7.4 with 15% 2-propanol and monitored at λ280nm using Thermo DAD-3000 RS Rapid Separation Diode Array Detector.

[0389] ADCs are purified by Size Exclusion Chromatography (SEC) and concentrated by using ultra centrifugation. To separate the antibody drug conjugates from the reaction mixture, preparative SEC purifications are performed using the ÄKTA instrument from GE Healthcare, on a Superdex® 200 increase 10/300 GL (1.0×30cm) column, at the flow rate of 0.6 mL/min eluting with BupH at pH 7.4 and monitored at λ280nm. To concentrate the product Amicon ® Ultra-4 Centrifugal Filters (Ultracel-10K) are used in Allegra x-12r centrifuge, and the solution is stirred after each concentration to avoid high aggregation.

Example 10**C**: CACNG1 androgen reporter assay experimental procedure

[0390] CACNG1 androgen reporter assay is described following, including relevant cell lines, proteins, reagents, and instrument type and model. CACNG1 is the γ1 subunit of the dihydropyridine receptor that is expressed specifically in skeletal muscle. Therefore, antibodies generated against CACNG1 could be used to deliver conjugated therapeutic payloads specifically to skeletal muscle to enhance therapeutic efficacy in muscle and reduce off-target toxicity. Conjugation of the potent metabolite of testosterone, dihydrotestosterone (DHT), to CACNG1 antibodies, may allow for androgen receptor signaling in muscle, leading to increased muscle mass and function. Here, CACNG1 antibodies conjugated to a linker with DHT payload were tested in an androgen receptor (AR) reporter cell line to determine whether these antibody conjugates can specifically activate the AR in CACNG1-expressing cells in vitro.

[0391] To assess signaling via the AR, LNCaP cells were transfected with lentivirus (Qiagen; ARE.Luc Cignal Lenti) to generate a stable cell line that expressed AR-luciferase reporter

(AR.Luc). A subset of these selected cells was transduced to express human CACNG1 and further selected, with this cell line being referred to as hCACNG1.AR.Luc.

[0392] For the bioassay, AR.Luc or hCACNG1.AR.Luc cells were plated at 5,000 cells/well in OptiMEM and 0.5% charcoal-stripped FBS in PDL-coated 96-well plates. Cells were then incubated for 24, 48, or 27 hours with CACNG1 antibodies or isotype control antibody conjugated to DHT via a linker, or payload DHT alone. All antibodies were conjugated with DHT at a drug-antibody-ratio (DAR) of ~4. After the respective timepoints, cells were lysed and incubated with One-GLO buffer, and luminescence was read on an Envision plate reader. Relative luminescence units (RLU) were plotted against log concentration in mol/L, adjusted for DAR.

Results summary and conclusions

[0393] Unconjugated DHT activated AR in both AR.Luc and hCACNG1.AR.Luc cell lines, whereas DHT conjugated to an isotype control antibody Ab 1 did not activate AR in either of these cell lines. Several CACNG1 antibody-DHT conjugates activated AR only in the hCACNG1.AR.Luc cell line, but not in the AR.Luc cell line. While the efficacy and potency of AR activation of CACNG1 antibody-DHT conjugates was lower than that of unconjugated DHT at 24 hours following treatment, activation of the AR was sustained at 48 and 72 hours with these conjugates, whereas unconjugated AR signal decreased substantially at these timepoints. Overall, these data demonstrate that conjugation of DHT to CACNG1 antibodies allows for specific activation of the AR in cells expressing hCACNG1, and that DHT conjugated to CACNG1 antibodies maintains sustained AR signaling over several days in hCACNG1-expressing cells in vitro.

Example 10D: Cell Free Assay For DHT Binding Affinity To AR

[0394] DHT has IC_{50} of 1.43 nM in AR assay performed by Thermo Fisher Scientific's SelectScreen™ Profiling Service.

AR (androgen receptor) - Antagonist Screen, Activated by R1881 (metribolone or methytrienolone, an AR agonist)

[0395] AR-UAS-bla GripTite™ 293 cells are thawed and prepared as described above for the Agonist Screen (see Example 5). 4 µL of a 10X serial dilution of Cyproterone Acetate (control antagonist starting concentration, 3,160 nM) or compounds are added to appropriate wells of a

Poly-D-Lysine assay plate. 32 µL of cell suspension is added to the wells and pre-incubated at 37°C/5% CO2 in a humidified incubator with compounds and control antagonist titration for 30 minutes. 4 µL of 10X control agonist R1881 at the pre-determined EC80 concentration is added to wells containing the control antagonist or compounds. The plate is incubated for 16-24 hours at 37°C/5% CO2 in a humidified incubator. 8 µL of 1 µM Substrate Loading Solution is added to each well and the plate is incubated for 2 hours at room temperature. The plate is read on a fluorescence plate reader. The results of the screen are shown in **Figure 12**.

EXAMPLE 10E: Plasma Stability OF ACACNG1 DHT-ADCS

[0396] To determine the plasma stability of CACNG1-DHT ncADC anti-hCACNG1 Ab 5-L3 and anti-hCACNG1 Ab 5- [L2] bearing DHT payload, ncADCs were incubated *in vitro* with the plasma from different species and the drug to antibody ratio (DAR) was evaluated.

[0397] The ncADC solution was spiked separately into pooled mouse, rat, cynomolgus monkey, or IgG depleted human plasma (Bipoint) to a final concentration of 50 µg/mL, and subsequently incubated at 37°C on ThermoMixer C (Eppendorf, Cat# 2231000574). A 100-µL aliquot was removed at time 0, 24, 48, 72 and 168 hours, and then immediately stored frozen at -80°C until the analysis.

[0398] For DAR analysis, ncADC was purified from plasma samples by immunoaffinity capture using a KingFisher Apex automated processor (Thermo Scientific, Cat#5400930).
First, biotinylated anti-human Fc antibody (Regeneron generated reagent) was immobilized on Dynabeads M280 streptavidin beads (Invitrogen, Cat#60210). Each plasma sample containing the ncADC was mixed at 950 rpm with 0.5 mg of the beads (Regeneron generated reagent coupled bead) at room temperature for 1.5 hours with gentle shaking. The beads were then washed three times with 500 µL of HBS-EP pH 7.4 buffer (GE Healthcare, Cat#BR100188), once with 500 µL water and once with 500 µL of 10% acetonitrile (VWR Chemicals, Cat#BDH83640.100E) in water. Following the washes, the ncADC were eluted by incubating the beads with 70 µL of 1% formic acid in 30:70 acetonitrile:water (v/v) for 15 minutes at room temperature. 50 µL eluted samples were further reduced by adding 50 µL 10 mM TCEP (Sigma, Cat 646547-10X1ML) and incubated at 37°C for 20 min in ThermoMixer C.

[0399] The reduced ncADC samples were injected onto a 1x50 mm 1.7 µm BEH300 C4 column (Waters, Cat# 186005589) for the separation and then detected by Synapt G2-Si Mass Spectrometer (Waters, Milford, MA). The flow rate was 80 µL/min (mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile). HPLC gradient eluted ncADC between 2.0-6.5 minute corresponding to 25-40 % of mobile phase B. The acquired spectra were deconvoluted using MaxEnt1 software with the following parameters: Mass range: 20-60 kDa; *m/z* range: 800-2500 Da; Resolution: 1.0 Da/channel; Width at half height: 0.7 Da; Minimum intensity ratios: 33%; Iteration max: 12.

Results summary and conclusions:

[0400] No significant change in DAR was observed for both ncADCs after the 168-hour incubation in rat, cynomolgus monkey or IgG depleted human plasma. Approximate 65% and 15% DAR loss in mouse plasma was observed for anti-hCACNG1 Ab 5-L2 and anti-hCACNG1 Ab 5-L3, respectively.

[0401] Tabulated Data Summary is presented in Table 7, below, and in **Figures 13-17**. **Figure 16** shows the level of androgen receptor (AR) activation in terms of relative light units (RLU; y-axis) after a 24 hour incubation of an LNCaP cell line modified to express luciferase upon androgen receptor activation (AR.Luc) with: dihydrotestosterone (DHT) alone (unconjugated DHT); an anti-hCACNG1 antibody (anti-hCACNG1 Ab 6, anti-hCACNG1 Ab 7, anti-hCACNG1 Ab 8, anti-hCACNG1 Ab 9, anti-hCACNG1 Ab 10 or anti-hCACNG1 Ab 5) conjugated via a VC-PAB linker to DHT (L2); or an anti-FelD isotype control antibody (Isotype control Ab 1) conjugated via a VC-PAB linker to DHT (L2); at varying concentrations (Log[Conc. (M)]; x-axis). **Figure 17** shows the level of androgen receptor (AR) activation in terms of relative light units (RLU; y-axis) after a 24 hour, 48 hour, or 72 hour incubation of a CACNG1 expressing LNCaP cell line modified to also express luciferase upon androgen receptor activation (hCACNG1.AR.Luc) with: dihydrotestosterone (DHT) alone (unconjugated DHT); an anti-hCACNG1 antibody (anti-hCACNG1 Ab 6, anti-hCACNG1 Ab 7, anti-hCACNG1 Ab 8, anti-hCACNG1 Ab 9, anti-hCACNG1 Ab 10 or anti-hCACNG1 Ab 5) conjugated via a VC-PAB linker to DHT (L2); or an anti-FelD isotype control antibody (Isotype control Ab 1) conjugated via a VC-PAB linker to DHT (L2); at varying concentrations (Log[Conc. (M)]; x-axis).

Table 7. DAR value anti-hCACNG1 Ab 5-L2 and anti-hCACNG1 Ab 5-L3 after the incubation in plasma

	anti-hCACNG1 Ab 5-L2				anti-hCACNG1 Ab 5-L3			
Time (Day)	Mouse	Rat	Monkey	Human	Mouse	Rat	Monkey	Human
0	3.95	3.94	3.94	3.94	3.83	3.83	3.88	3.84
1	3.24	3.94	3.95	3.95	3.69	3.82	3.84	3.84
2	2.81	3.95	3.95	3.95	3.56	3.81	3.84	3.84
3	2.39	3.95	3.94	3.94	3.47	3.79	3.83	3.84
7	1.74	3.95	3.93	3.94	3.27	3.74	3.85	3.85

* * *

[0402] As various changes can be made in the above-described subject matter without departing from the scope and spirit of the present invention, it is intended that all subject matter contained in the above description, or defined in the appended claims, be interpreted as descriptive and illustrative of the present invention. Many modifications and variations of the present invention are possible in light of the above teachings. Accordingly, the present description is intended to embrace all such alternatives, modifications, and variances which fall within the scope of the appended claims.

[0403] All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference in their entirety as if physically present in this specification.

WHAT IS CLAIMED:

1. An antibody-drug conjugate comprising an antigen-binding protein that specifically binds to Human Calcium Voltage Gated Channel Auxiliary Subunit Gamma 1 (hCACNG1), wherein the antigen-binding protein is conjugated directly or via a linker to at least one therapeutic agent, or a pharmaceutically acceptable salt thereof, wherein the antibody-drug conjugate has a structure according to Formula (I):

   $A - [L - P]_y$ (I),

   wherein A is the antigen-binding protein;

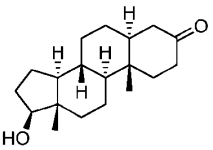
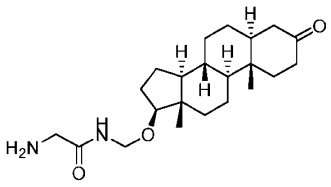
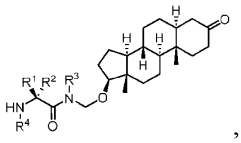
   L is absent or a linker;

   P is a therapeutic agent, and

   y is an integer from 1 to 8.

2. The antibody-drug conjugate of claim 1, wherein the antigen-binding protein is an anti-hCACNG1 antibody or antigen-binding fragment thereof that comprises a set of HCDR1-HCDR2-HCDR3-LCDR1-LCDR2-LCDR3 amino acid sequences selected from the group consisting of SEQ ID NOs: 4-6-8-12-AAS-16, SEQ ID NOs: 20-22-24-26-28-ATS-32, SEQ ID NOs: 36-38-40-44-KAS-48, SEQ ID NOs: 52-54-56-60-GAS-64; SEQ ID NOs: 68-70-72-76-AAS-80; SEQ ID NOs: 84-86-88-92-AAS-96; SEQ ID NOs: 100-102-104-108-AAS-112; SEQ ID NOs: 116-118-120-124-GA-128; SEQ ID NOs: 132-134-136-140-GAS-144; SEQ ID NOs: 148-150-152-156-RN-160; SEQ ID NOs: 164-166-168-172-DNN-176; SEQ ID NOs: 180-182-186-188-GAS-192; SEQ ID NOs: 36-297-299-303-YNS-305; and SEQ ID NOs: 316-318-152-324-RNN-326.
3. The antibody-drug conjugate of any one of claims 1-2, wherein the anti-hCACNG1 antibody or antigen-binding fragment thereof comprises an HCVR/LCVR amino acid sequence pair having at least 90% sequence identity to an HCVR/LCVR amino acid sequence pair selected from the group consisting of SEQ ID NOs: 2/10, SEQ ID NOs: 18/26, SEQ ID NOs: 34/42, SEQ ID NOs: 50/58, SEQ ID NOs: 66/74, SEQ ID NOs: 82/90, SEQ ID NOs: 98/106, SEQ ID NOs: 114/122,

SEQ ID NOs: 130/138, SEQ ID NOs: 146/154, SEQ ID NOs: 162/170, SEQ ID NOs: 178/186, SEQ ID NOs: 294/301, and SEQ ID NOs: 314/322.

4. The antibody-drug conjugate of any one of claims 1-3, wherein the therapeutic agent is dihydrotestosterone (DHT), or a prodrug or derivative thereof.
5. The antibody-drug conjugate of any one of claims 1-3, wherein the therapeutic agent is selected from the group consisting of DHT having the structure  and proDHT having the structure selected from the group consisting of  and  ,

wherein R_1 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

R_2 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

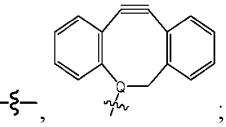
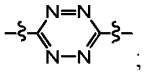
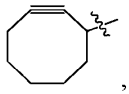
R_3 is selected from the group consisting of H and a C_{1-3} alkyl, and

R_4 is selected from the group consisting of H and a C_{1-3} alkyl.

6. The antibody-drug conjugate of any one of claims 1-3, wherein n is 2 or 4.
7. The antibody-drug conjugate of any one of claims 1-6, wherein the linker has the structure:

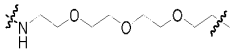
-L1-B-L2- , wherein:

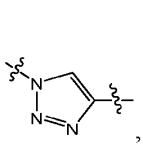
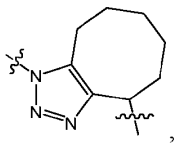
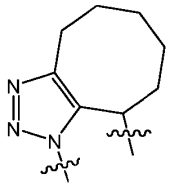
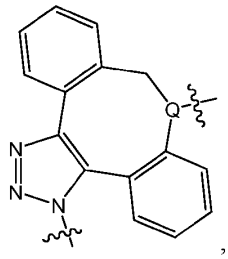
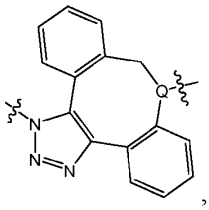
L1 is a first linker unit covalently attached to the antigen-binding protein;

B is absent or a unit comprising at least one adduct of group B', where the group B' is selected from -N_3,  ,  ; and  , where Q is C or N;

L2 is absent or a second linker unit covalently attached to the unit B via at least one group B", wherein the group B' and the group B" form the at least one adduct,

provided that when B is absent, L2 is also absent and L1 is covalently attached to the therapeutic agent, and when L2 is present, L2 is covalently attached to the therapeutic agent.

8. The antibody-drug conjugate of claim 7, wherein the L1 comprises C_{1-6} alkyl, phenyl, -NH-, -C(O)-, -$(CH_2)_u$-NH-C(O)-, -$(CH_2)_u$-C(O)-NH-, -$(CH_2$-CH_2-$O)_v$-, -$(CH_2)_u$-$(O$-CH_2-$CH_2)_v$-C(O)-NH-, a peptide unit comprising from 2 to 4 amino acids, or combinations thereof, each of which may be optionally substituted with one or more of -S-, -$S(O_2)$-, -C(O)-, -$C(O_2)$-; and CO_2H, wherein subscripts u and v are independently an integer from 1 to 8.
9. The antibody-drug conjugate of claim 8, wherein the L1 is  .
10. The antibody-drug conjugate of any one of claims 7-9, wherein the B has a structure selected from the group consisting of:

 ,  ,  ,  , and  ,

wherein Q is C or N.

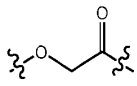
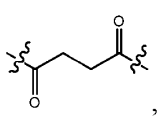
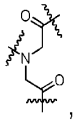
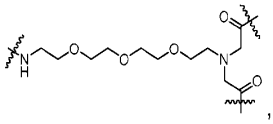
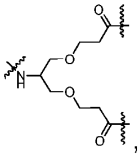
11. The antibody-drug conjugate of any one of claims7-10, wherein the L2 has a structure:

-SP1-AA-SP2- (L2), wherein:

SP1 is absent or a first spacer unit;

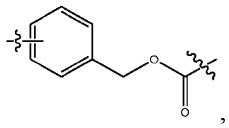
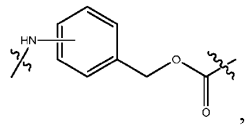
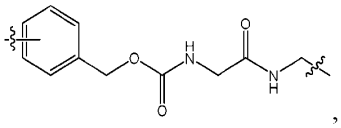
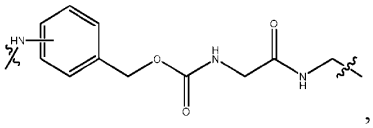
AA is absent or a peptide unit comprising from 2 to 4 amino acids;

SP2 is absent or a second spacer unit covalently attached to the therapeutic agent.

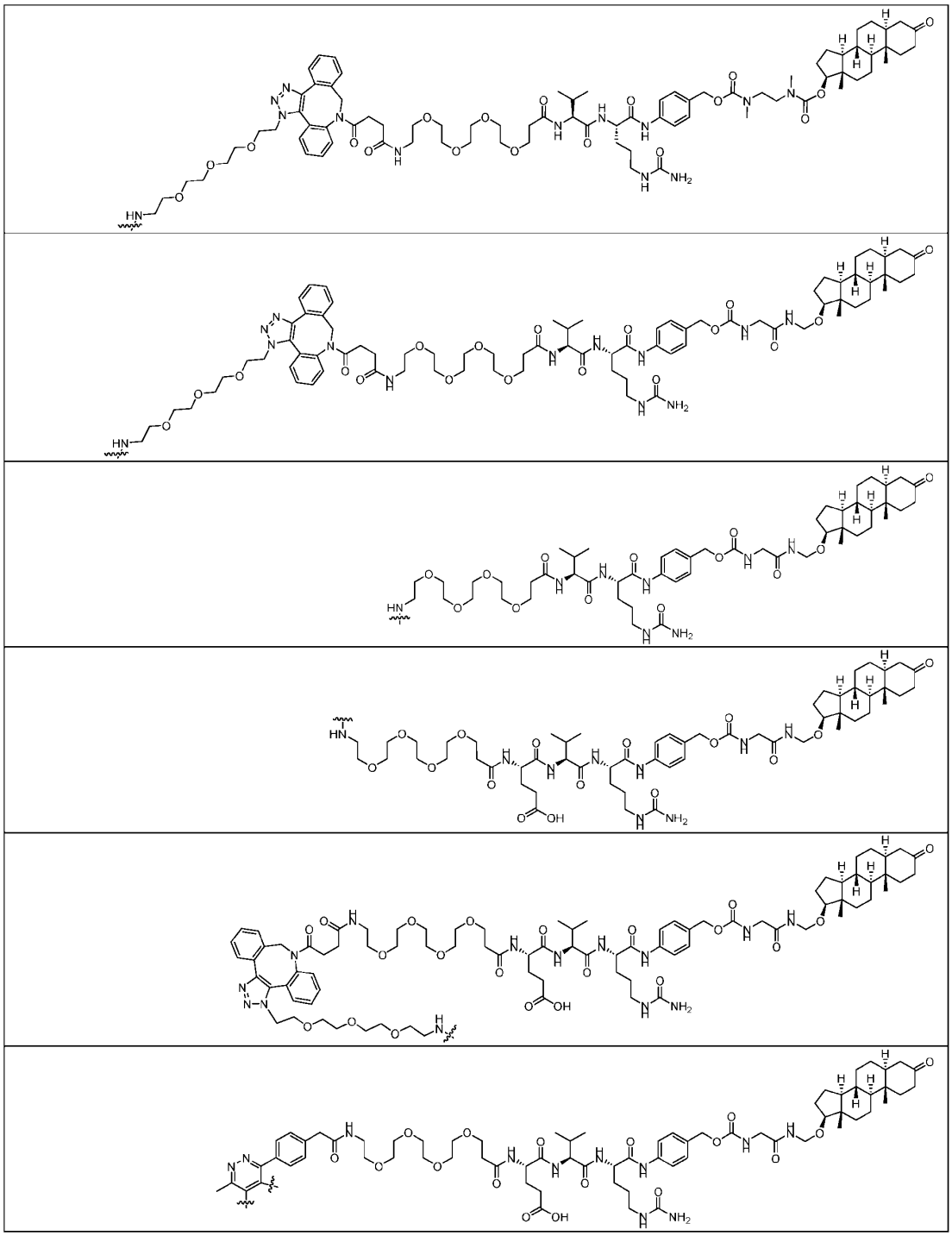
12. The antibody-drug conjugate of claim 11, wherein the SP1 is absent or selected from the group consisting of  ,  ,  ,  ,  , C_{1-6} alkyl, $-(CH_2-CH_2-O)_v-$, $-(CH_2-CH_2-O)_v-(CH_2)_u-$, $-(CH_2-CH_2-O)_v-(CH_2)_u-C(O)$, $-O-CH_2-C(O)-NH$, $-O-CH_2-C(O)-NH-(CH_2-CH_2-O)_v-(CH_2)_u-$, $-O-CH_2-C(O)-NH-(CH_2-CH_2-O)_v-(CH_2)_u-C(O)-NH$, $-NH-$, $-C(O)-$, $-NH-C(O)-$, $-NH-(CH_2)_u-$, $-NH-(CH_2)_u-C(O)-$, $-NH-(CH_2-CH_2-O)_v-$, $-NH-(CH_2-CH_2-O)_v-C(O)-$, $-NH-(CH_2-CH_2-O)_v-(CH_2)_u-$, $-NH-(CH_2-CH_2-O)_v-(CH_2)_u-C(O)-$, $-(CH_2)_u-NH-C(O)-$, $-NH-(CH_2)_u-NH-C(O)-$, $-NH-(CH_2)_u-C(O)-NH-$, or combinations thereof; wherein subscripts u and v are independently an integer from 1 to 8.

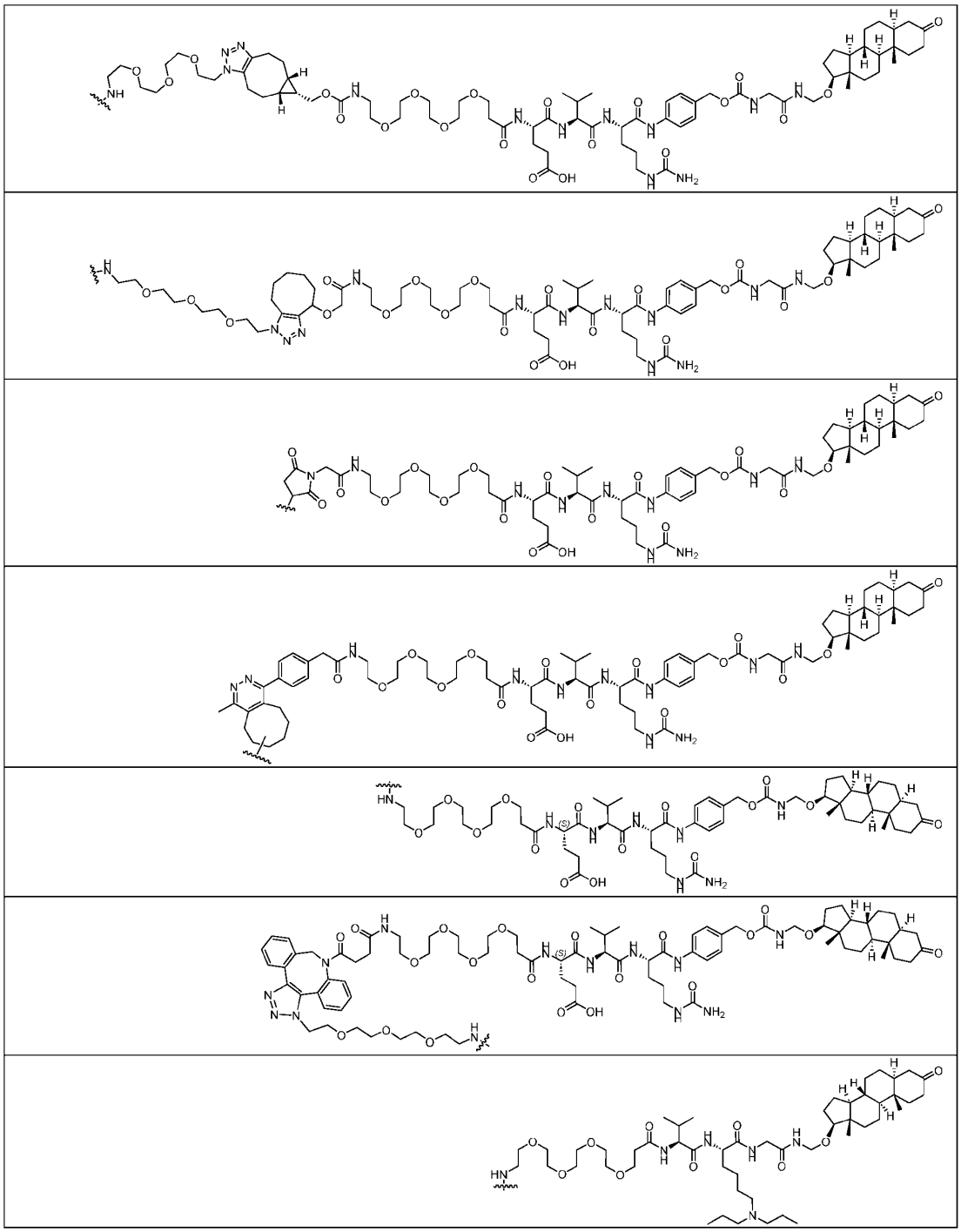
13. The antibody-drug conjugate of claim 11 or claim 12, wherein the AA is a peptide unit comprising from 2 to 4 amino acids selected from glycine, valine, phenylalanine, proline, glutamic acid, lysine, N,N-dipropyl lysine, phenylalanine, and citrulline, and combinations thereof.

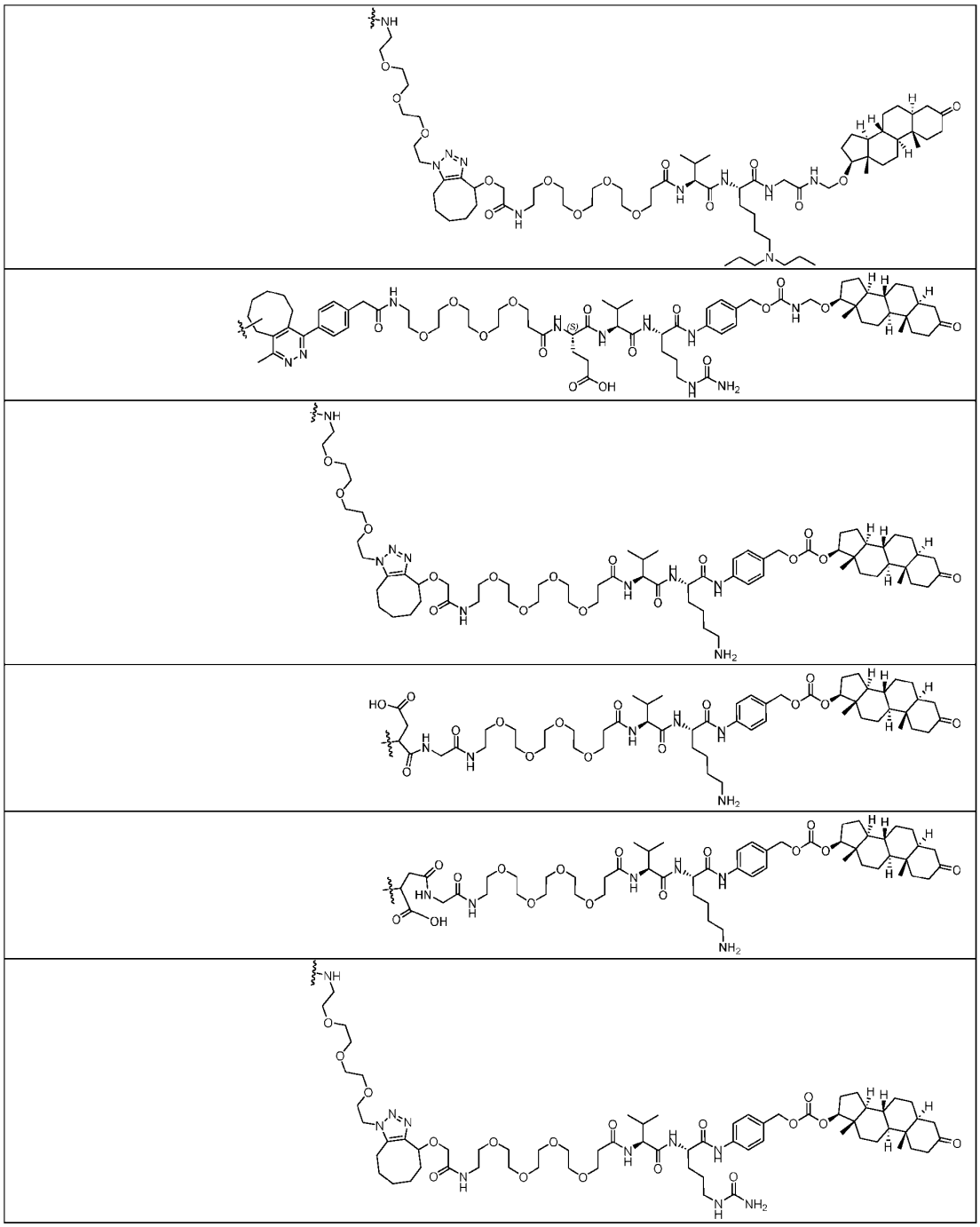
14. The antibody-drug conjugate of claim 13, wherein the AA is valine-citrulline, valine-alanine, valine-lysine, valine-N,N-dipropyl lysine, phenylalanine-lysine, glycine-glycine-glycine (GGG), glycine-glycine-glycine-glycine (GGGG (SEQ ID NO: 289)), glycine-glycine-phenylalanine (GGF), glycine-glycine-phenylalanine-glycine (GGFG (SEQ ID NO: 290)), L-glutamic acid-valine-citrulline (^{L}EVC), and D-glutamic acid-valine-citrulline (^{D}EVC).

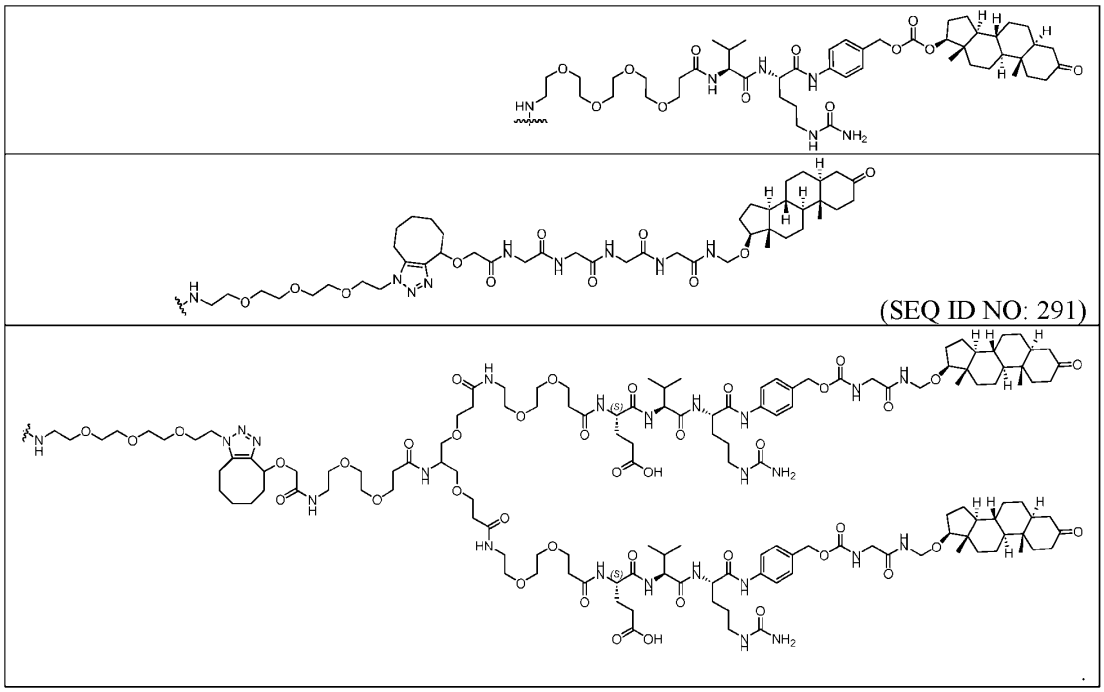
15. The antibody-drug conjugate of any one of claims 11-14, wherein the SP2 is absent or selected from the group consisting of  ,  ,  ,  , and combinations thereof.

16. The antibody-drug conjugate of any one of claims 1-15, wherein the linker-therapeutic agent (L-P) has a structure selected from the group consisting of:



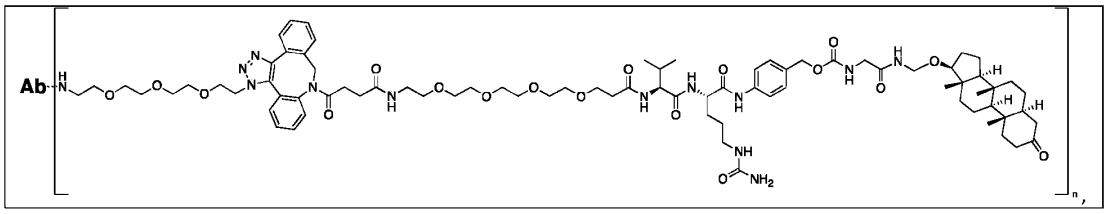


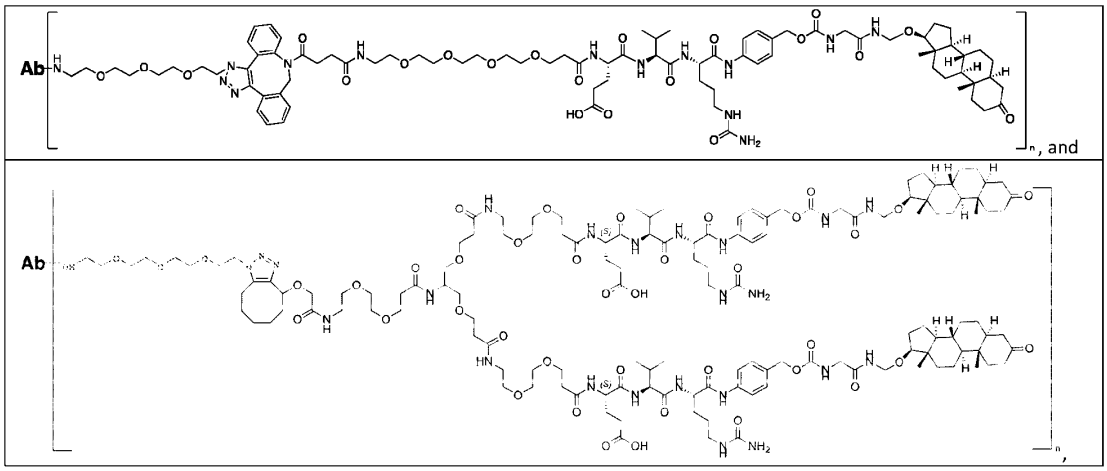




(SEQ ID NO: 291)

17. The antibody-drug conjugate of any one of claims 1-16, wherein the linker is attached to the side chain of a glutamine residue of the the antigen-binding protein.
18. The antibody-drug conjugate of claim 17, wherein the glutamine residue is naturally present in a CH2 or CH3 domain of the antigen-binding protein.
19. The antibody-drug conjugate of claim 17, wherein the glutamine residue is introduced to the antigen-binding protein by modifying one or more amino acids.
20. The antibody-drug conjugate of any one of claims 1-19, wherein the antibody-drug conjugate has a structure selected from the group consisting of:





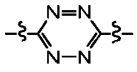
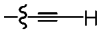
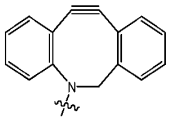
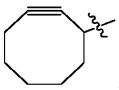
wherein Ab is the antigen-binding protein and n is an integer from 1 to 4.

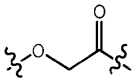
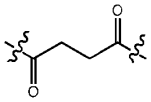
21. A compound according to Formula (L2-P) or (L2'-P):

B"-SP1-AA-SP2-P (L2-P),

H_2N-SP1-AA-SP2-P)$_p$ (L2'-P),

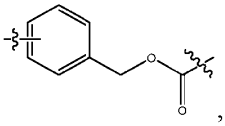
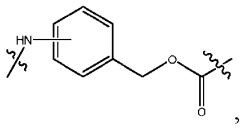
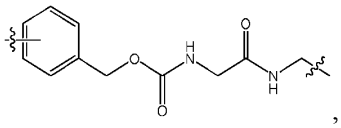
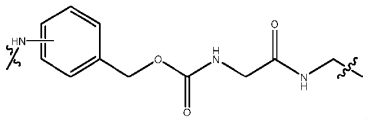
or a pharmaceutically acceptable salt thereof, wherein:

B" is selected from the group consisting of -N_3,  ,  ,  and  ;

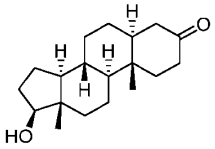
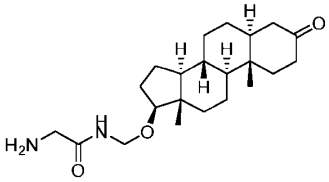
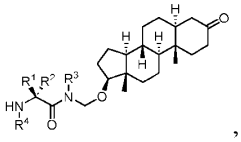
SP1 is absent or a first spacer unit selected from the group consisting of  and  ; a $C_{1\text{-}6}$ alkyl, -$(CH_2$-CH_2-$O)_v$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O), -O-CH_2-C(O)-NH, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -O-CH_2-C(O)-NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-NH, -NH-, -C(O)-, -NH-C(O)-, -NH-$(CH_2)_u$-, -NH-$(CH_2)_u$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-, -NH-$(CH_2$-CH_2-$O)_v$-C(O)-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-, -NH-$(CH_2$-CH_2-$O)_v$-$(CH_2)_u$-C(O)-, -

$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-NH-C(O)-, -NH-$(CH_2)_u$-C(O)-NH-, or combinations thereof; wherein subscripts u and v are independently an integer from 1 to 8;

AA is absent or a peptide unit comprising from 2 to 4 amino acids;

SP2 is absent or a second spacer unit selected from the group consisting of  ,  ,  ,  , and combinations thereof,

and

P is a therapeutic agent selected from the group consisting of DHT having the structure  and proDHT having the structure selected from the group consisting of  and  ,

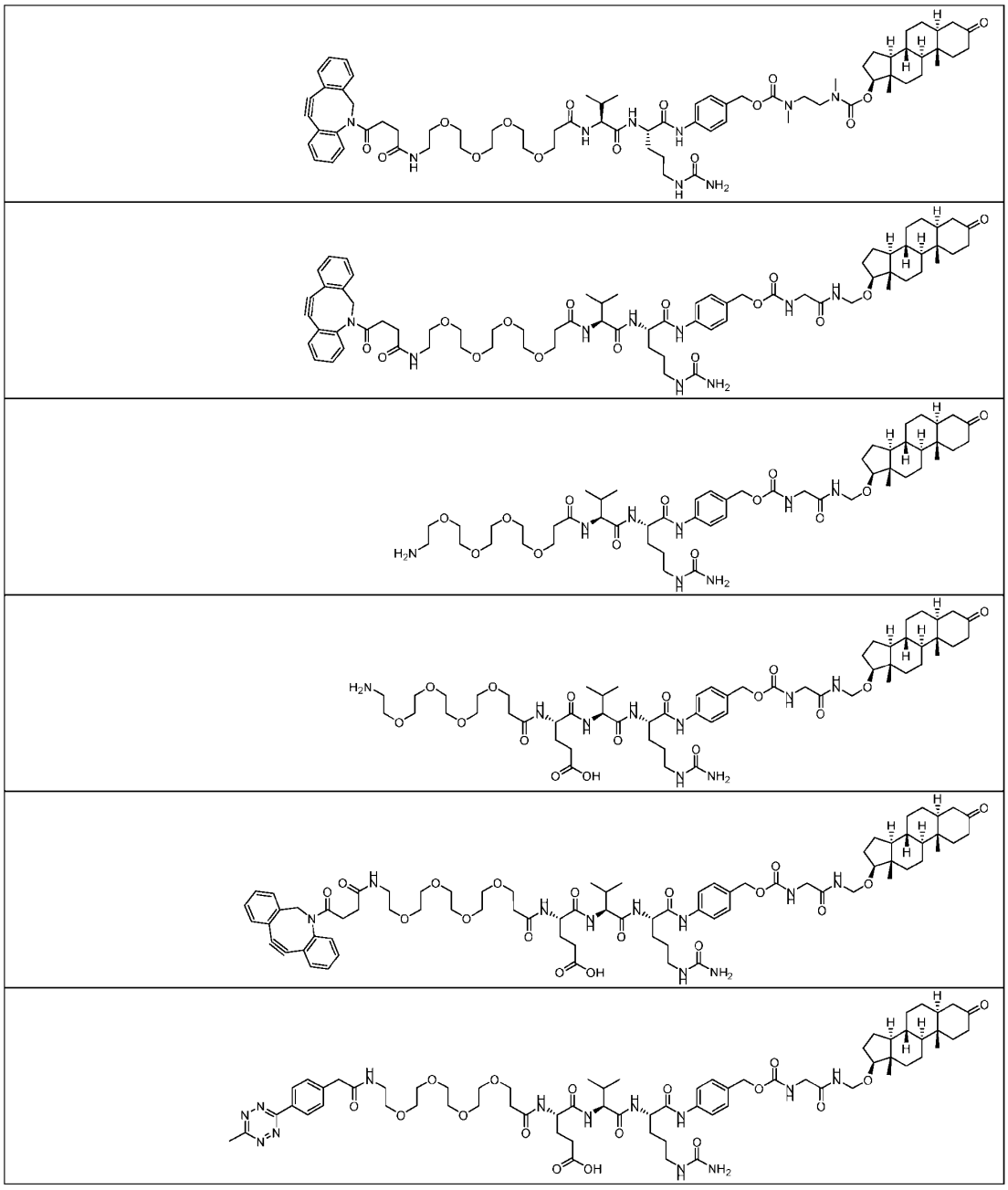
wherein R_1 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

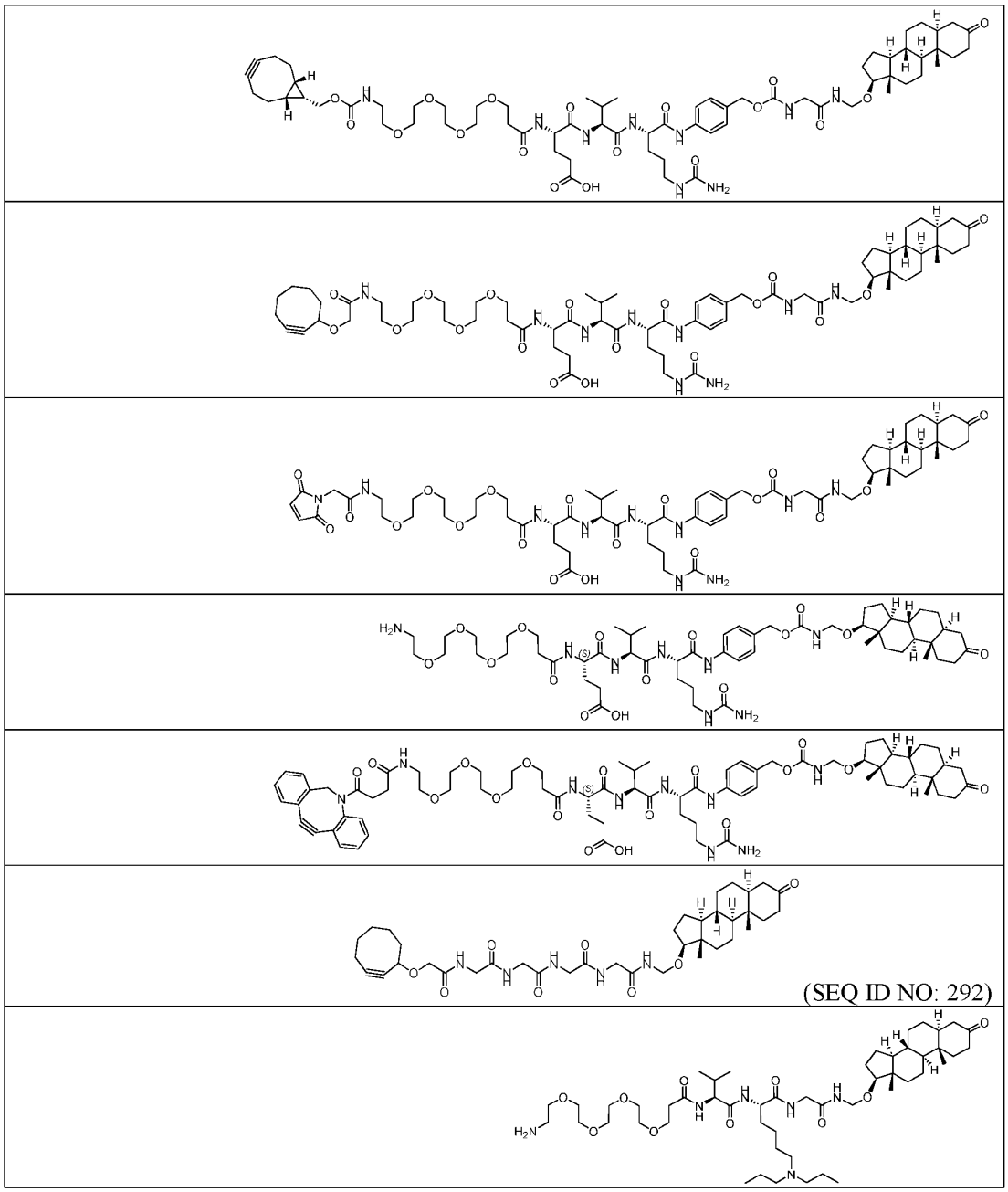
R_2 is selected from the group consisting of H, C_{1-6} alkyl, $(CH_2)_{0-6}$-OH, $(CH_2)_{0-6}$-NH_2, $(CH_2)_{0-6}$-NH-C_{1-3} alkyl, and $(CH_2)_{0-6}$-N-(C_{1-3} alkyl)$_2$;

R_3 is selected from the group consisting of H and a C_{1-3} alkyl, and

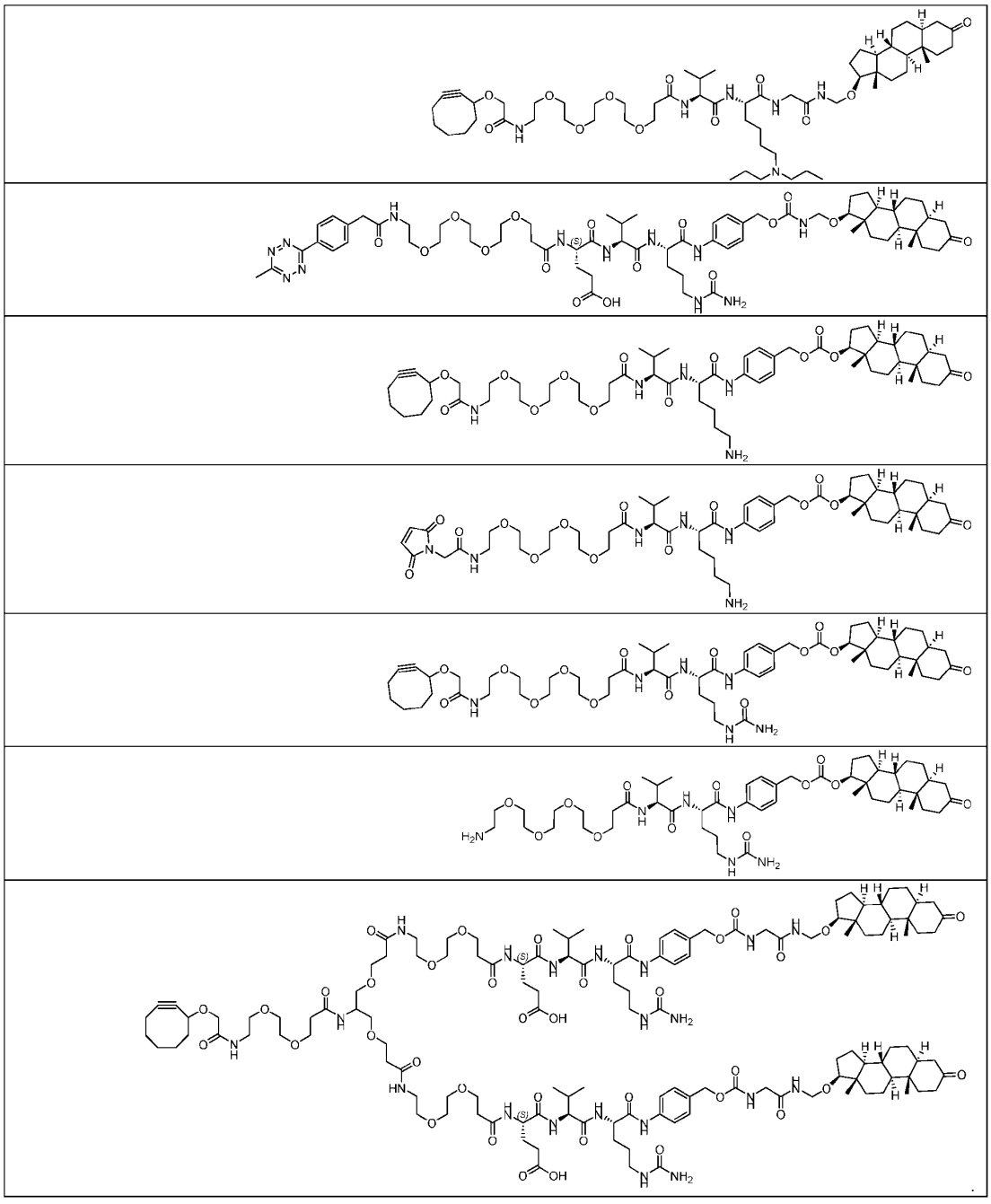
R_4 is selected from the group consisting of H and a C_{1-3} alkyl.

22. The compound of claim 21 having a structure selected from the group consisting of:



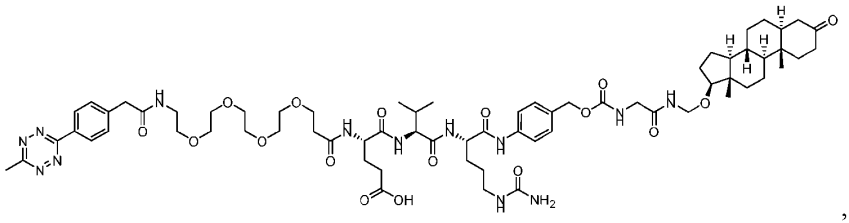


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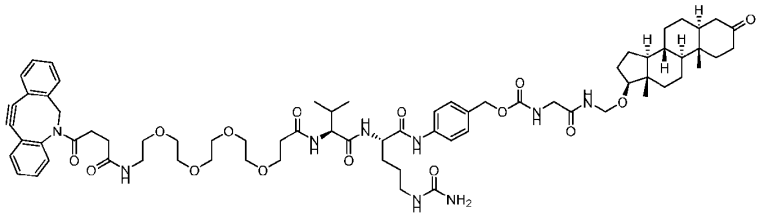


23. An antibody-drug conjugate or a pharmaceutically acceptable salt thereof comprising an antigen-binding protein that specifically binds to Human Calcium Voltage Gated Channel Auxiliary

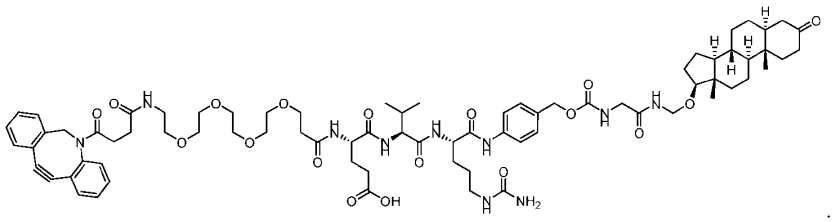
Subunit Gamma 1 (hCACNG1) conjugated to the linker-payload selected from the group consisting of



,



, and



.

24. A composition comprising a population of the antibody-drug conjugates of any one of claims 1-20 and 23, having a drug-antibody ratio (DAR) of about 0.5 to about 8.0.
25. The composition of claim 24 having a DAR of about 1.0 to about 2.5
26. The composition of claim 25 having a DAR of about 2.0.
27. The composition of claim 24 having a DAR of about 3.0 to about 4.5
28. The composition of claim 27 having a DAR of about 4.0.
29. A pharmaceutical composition comprising the antibody-drug conjugate of any one of claims 1-20 and 23 and a diluent, a carrier, and/or an excipient.
30. A pharmaceutical dosage form comprising the antibody-drug conjugate of any one of claims 1-20 and 23 or the pharmaceutical composition of claim 29.
31. A method of treating a condition in a subject in need thereof, wherein the method comprises administering to the subject the antibody-drug conjugate of any one of claims 1-20 and 23, the pharmaceutical composition of claim 29, or the pharmaceutical dosage form of claim 30.

32. The method of claim 31, wherein the condition is selected from the group consisting of muscle wasting and genetic muscle diseases.
33. The method of claim 32, wherein the condition is selected from the group consisting of adult spinal muscular atrophy, amyotrophic lateral sclerosis (ALS), anoctaminopathy, autoimmune neuropathy, Becker muscular dystrophy, Bethlem myopathy, calapainopathy, caveolinopathy, central core disease, Charcot-Marie-Tooth disease (CMT), congenital fibre type disproportination (CFTD), congenital muscular dystrophy, congenital myasthenic syndrome (CMS), congenital myopathy, congenital myotonic dystrophy, dermatomyositis (DM), disorders of the neuromuscular junction, distal myopathy with rimmed vacuoles (DMRV), Duchenne muscular dystrophy (DMD), Emery-Dreifuss muscular dystrophy, facioscapulohumeral muscular dystrophy (FSHD), facioscapuloperoneal muscular dystrophy (FSPD), fibrodysplasia ossificans progressiva (FOP), glycogen storage disease type V (GSD V), GNE myopathy (GNEM), hereditary inclusion body myopathy (HIBM), hereditary inclusion body myopathy type 2 (HIBM 2), hereditary motor and sensory neuropathy (HMSN), hereditary neuropathies, inclusion body myositis (IBM), infantile progressive spinal muscular atrophy, inflammatory neuropathy, infectious myelitis, intermediate spinal muscular atrophy, juvenile dermatomyositis, juvenile spinal muscular atrophy, Landouzy-Dejerine, limb girdle muscular dystrophies (LGMDs), McArdle disease, merosin-deficient congenital muscular dystrophy, metabolic myopathy, minicore myopathy, multicor myopathy, mitochondrial myopathy, muscular dystrophy, myasthenia gravis, myofibrillar myopathy, myositis, myositis ossificans progressiva (MOP), myotonic dystrophy, myotubular and other centronuclear myopathy, nemaline myopathy, Nonaka myopathy, oculopharyngeal muscular dystrophy (OPMD), periodic paralysis, polymyositis (PM) and dermatomyositis (DM), quadriceps-sparing myopathy (QSM), sarcoglycanopathy, sarcopenia, SEPN1-related myopathy, spinal muscular atrophy (SMA), Steinert's disease, Ullrich congenital muscular dystrophy, VCP disease, and age-related progressive loss of muscle mass and strength.
34. A method of selectively delivering a compound into a cell, wherein the compound is the antibody-drug conjugate of any one of claims 1-20 and 23.
35. A method of selectively targeting an antigen on a surface of a cell with a compound, wherein the compound is according to any one of claims 1-20 and 23.
36. The method of claim 35, wherein the cell is a mammalian cell.

37. The method of claim 35 or claim 36, wherein the cell is a human cell.
38. The method of any one of claims 35-37, wherein the cell is a muscle cell.
39. The method of any one of claims 31-38, wherein the antibody-drug conjugate is administered intramuscularly, intravenously, or subcutaneously.
40. A method of causing internalization of a compound by a myofiber, the method comprising contacting the myofiber with the antibody-drug conjugate of any one of claims 1-20 and 23.

41. A method of producing a compound having a structure according to Formula (A):

$$Ab\text{-}(L1\text{-}B\text{-}L2\text{-}P)_n \quad (A),$$

or a pharmaceutically acceptable salt thereof, wherein:

Ab is an antigen-binding protein of any one of claims 1-14;

L1 is a first linker covalently bound to the side chain of a glutamine residue of the Ab;

B is a moiety comprising a triazole;

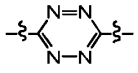
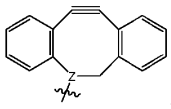
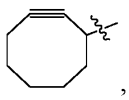
L2 is a second linker covalently bound to the therapeutic agent P;

P is a therapeutic agent selected from the group consisting of DHT and proDHT, and

n is an integer from 1 to 8, wherein the method comprises the steps of:

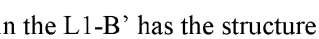
a) contacting, in the presence of a transglutaminase, the A comprising at least one glutamine residue with at least one compound L1-B';

b) contacting the product of step a) with one or more equivalents of a compound B"-L2-P, wherein the group B" is capable of covalently attaching to the group B',

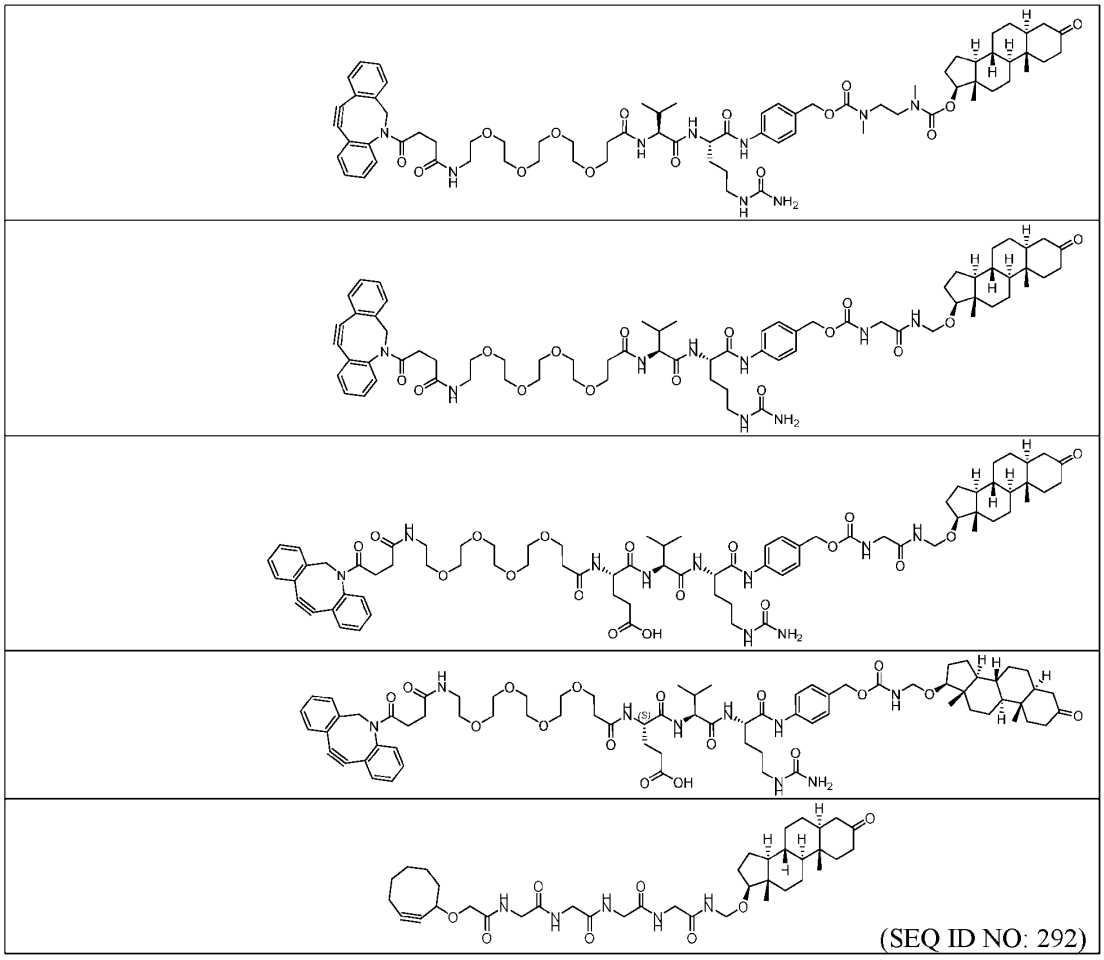
wherein one of the groups B' and B" is selected from $-N_3$ and  ; and the other of the groups B' and B" is selected from ,  ; and  , where Z is C or N; and

c) isolating the produced compound of Formula (A).

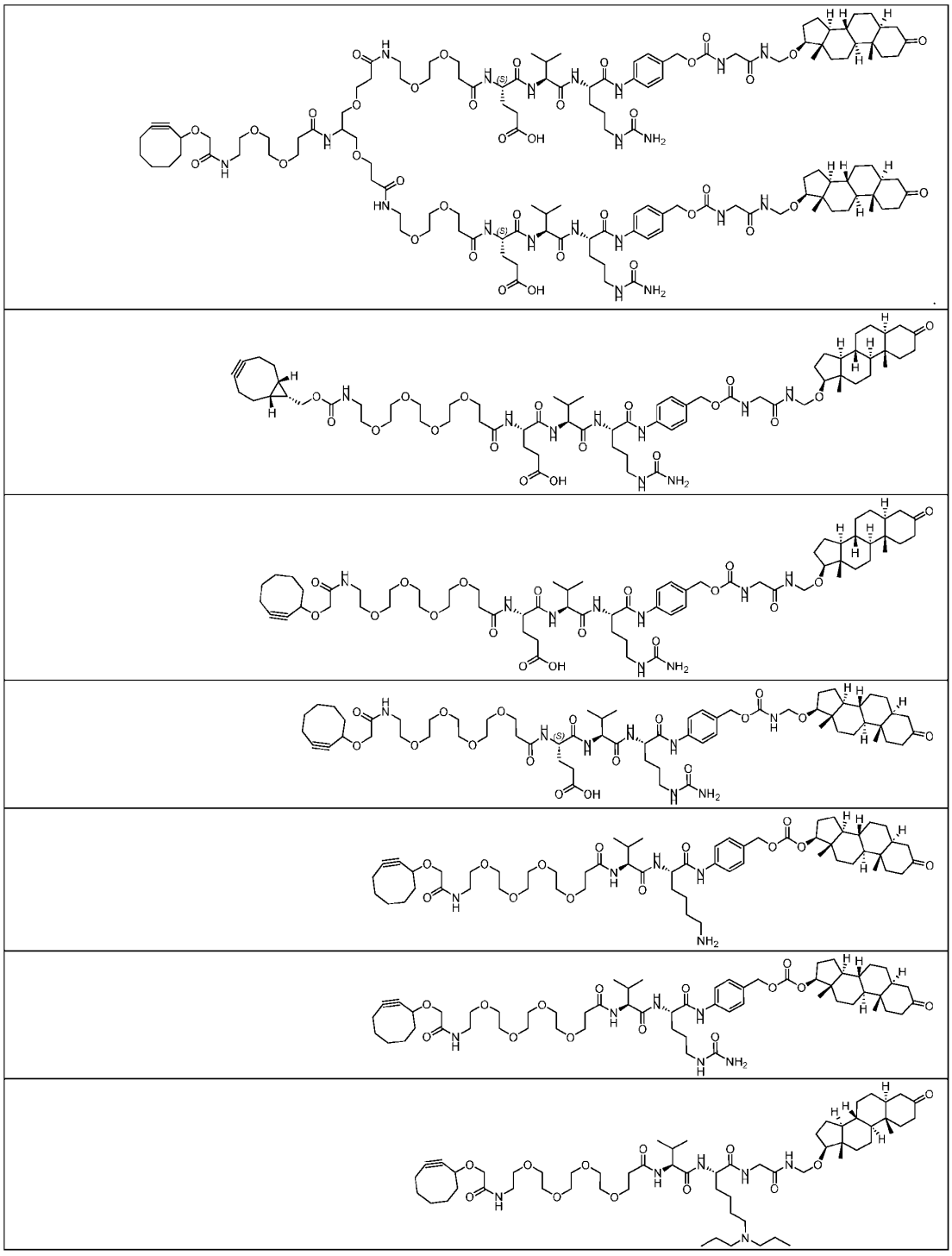
42. The method of claim 41, wherein the A has glutamine residues at positions 295 (Q295) and 297 (N297Q).

43. The method of claim 41, wherein the L1-B' has the structure  .

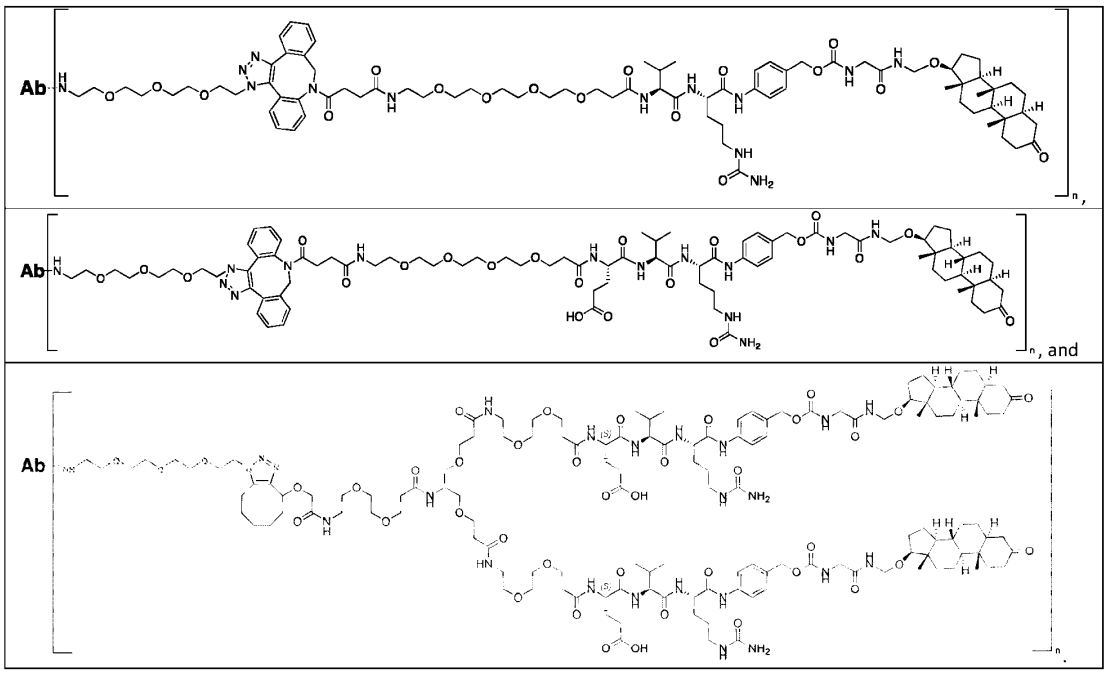
44. The method of claim 41, wherein the compound B"-L2-P has the structure selected from the group consisting of:



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45. The method of claim 41, wherein the compound of Formula (A) has the structure selected from the group consisting of:



46. A method of producing a compound having a structure according to Formula (I):

$A - [L - P]_y$ (I),

wherein A is an antigen-binding protein;

L is a linker;

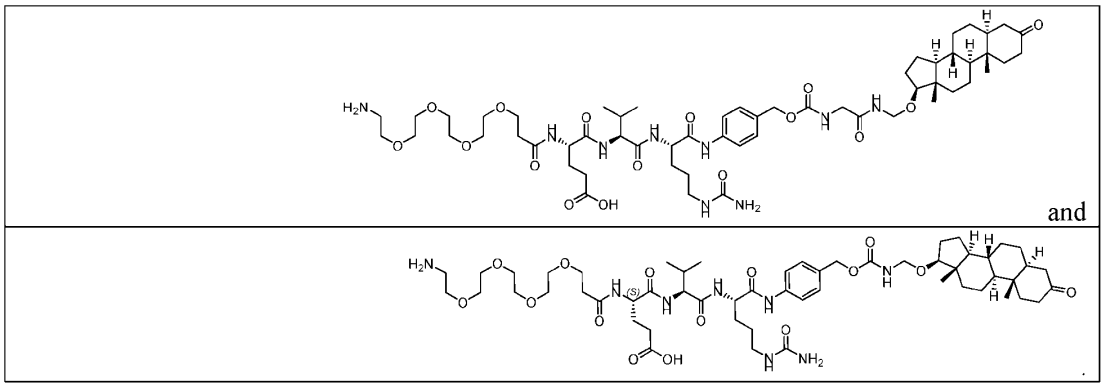
P is a therapeutic agent selected from the group consisting of DHT and proDHT, and

y is an integer from 1 to 8, wherein the method comprises the steps of:

a) contacting, in the presence of a transglutaminase, the A comprising at least one glutamine residue with at least one compound L-P, wherein the compound L-P has at least one terminal amine moiety, and

b) isolating the produced compound of Formula (I).

47. The method of claim 46, wherein the compound L-P has the structure selected from the group consisting of:



and