LYSOSOMAL TARGETING BIFUNCTIONAL MOLECULES FOR DEGRADATION OF THYROID STIMULATING HORMONE RECEPTOR AUTOANTIBODIES

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Patent Application No. 63/571,393, filed March 28, 2024, the content of which is incorporated herein by reference in its entirety in the present disclosure.

BACKGROUND

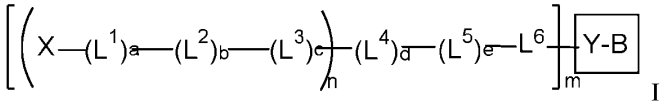
Many therapeutics act by binding a functionally important site on a target protein, thereby modulating the activity of that protein, or by recruiting immune effectors, as with many monoclonal antibody drugs, to act upon the target protein. However, there is an untapped reservoir of medically important human proteins that are considered to be "undruggable" because these proteins are not readily amenable to currently available therapeutic targeting approaches.

Graves' disease is an autoimmune disease that primarily affects the thyroid gland and is caused by autoantibodies to the thyroid-stimulating hormone receptor (TSHR). (T.F. Davies et al., Graves' Disease, Nature Reviews - Disease Primers, 6:52 (2020)). Graves' Disease, also known as von Basedow disease, is characterized by an enlarged and overactive thyroid gland (Graves' hyperthyroidism). Other symptoms include arrythmia, goiter, irritability, fatigue, weight-fluctuation, bone loss; extrathyroidal manifestations including orbitopathy (thyroid eye disease, TED), and pre-tibial skin thickening or rash. Graves' Disease affects approximately 2% of women and 0.2% of men globally. Current treatments include antithyroid drugs, radioiodine and surgery. The treatment of patients with GD has not changed in many years and newer, more effective therapies could improve outcomes. Thyroid eye disease (TED), also known as thyroid-associated orbitopathy, Graves ophthalmopathy and Graves orbitopathy, is an autoimmune condition that can be disfiguring and impair sight. (Kossler et al.) In the United States, TED affects approximately 16 of every 100,000 females and 3 of every 100,000 males. Improved treatments over the current standards of care for both Graves' disease and TED are needed.

LYTACs (lysosomal targeting chimera) are bifunctional molecules for selective protein degradation. The first LYTACs targeted extracellular proteins for degradation via engaging the cation-independent mannose-6-phosphate receptor (CI-M6PR) or the asialoglycoprotein receptor (ASGPR). ASGPR is the transmembrane glycoprotein receptor found primarily in hepatocytes which plays an important role in serum glycoprotein homeostasis by mediating the endocytosis and lysosomal degradation of glycoproteins with exposed terminal galactose or N-acetylgalactosamine (GalNAc) residues.

SUMMARY

The present disclosure provides lysosomal targeting bifunctional molecules (LYTACs, also referred to as conjugates) that target disease-causing anti-TSHR autoantibodies for degradation. The lysosomal targeting bifunctional molecules include a ligand moiety that specifically binds to an asialoglycoprotein receptor (ASGPR), and TSHR autoantigen that binds pathogenic autoantibodies. In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula I:



I

or a prodrug thereof, or a salt thereof, wherein:

n is 1 to 10;

m is 1 to 10;

X is a moiety that binds to asialoglycoprotein receptor (ASGPR);

Y is a carrier polypeptide connected to B;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody;

each L^1 to L^6 is independently a linking moiety which together provide a linear or branched linker between each X and the Y-B complex; and

a, b, c, d, and e are each independently 1, 2, 3, 4, or 5.

In some embodiments, B comprises an extracellular domain of TSHR or a fragment thereof.

In some embodiments, B comprises a polypeptide having at least 80% (e.g., at least 85%, or at least 90%) sequence identity with a sequence of SEQ ID NOs: 1-7. In some embodiments, B comprises a polypeptide having at least 95% sequence identity with a sequence of SEQ ID NOs: 1-7. In some embodiments, B comprises a polypeptide having at least 98% sequence identity with a sequence of SEQ ID NOs: 1-7. In some embodiments, B comprises a polypeptide of one of SEQ ID NOs: 1-7. In some embodiments, B consists essentially of a polypeptide of one of SEQ ID NOs: 1-7. In some embodiments, B has one or more amino acid substitutions selected from R112P, D143P, D151E, V169R, C176S, K250Q, and I253R as compared to a sequence of SEQ ID NO: 1-7. In some embodiments, B has one or more amino acid substitutions selected from H63C, R112P, D143P, D151E, V169R, I253R as compared to a sequence of SEQ ID NO: 1-7.

In some embodiments, Y is selected from albumin, albumin binding domain, multimerization domain, Fc domain, Fc (monomer), Fc (dimer), fragments thereof (e.g., synthetic peptides), and variants thereof. In some embodiments, Y comprises an albumin, a fragment thereof, or a variant thereof. In some embodiments, Y comprises human serum albumin (HSA), an HSA domain, bovine serum albumin (BSA), a fragment thereof, or a variant thereof. In some embodiments, Y is an HSA variant engineered for increased stability, conjugation efficiency (Cys or Lys), and/or FcRn binding. In some embodiments, Y comprises a polypeptide having 1 to 10 amino acid substitutions, deletions or additions (for example, 1, 2, 3, 4 or 5 amino acid substitutions, deletions or additions) as compared to a sequence of SEQ ID NOs: 8-12, 14 and 15. In some embodiments, the 1 to 10 amino acid substitutions, deletions or additions are selected from C34A, V54C, K93C, H128C, K262C, and E294C. In some embodiments, Y comprises a site-specific mutation of a naturally occurring amino acid residue. In some embodiments, Y comprises a polypeptide having at least 80% (e.g., at least 90%, at least 95%, at least 98%) sequence identity with a sequence of SEQ ID NOs: 8-12, 14 and 15. In some embodiments, Y comprises a polypeptide of one of SEQ ID NOs: 8-12, 14 and 15. In some embodiments, Y comprises a Fc domain, a fragment thereof, or a variant thereof. In some embodiments, Y comprises Fc (monomer). In some embodiments, Y comprises Fc (dimer). In some embodiments, Y comprises a Fc domain engineered for increased stability, conjugation efficiency (Cys or Lys), and/or FcRn binding.

In some embodiments, Y comprises a polypeptide having 1 to 10 amino acid substitutions, deletions or additions (for example, 1, 2, 3, 4 or 5 amino acid substitutions, deletions or additions) as compared to a sequence of SEQ ID NO: 15. In some embodiments, the 1 to 10 amino acid substitutions, deletions or additions are selected from T366W, T366S, X368A, A378C, X407V, and L443C. In some embodiments, Y comprises a polypeptide having at least 80% (e.g., at least 90%, at least 95%, at least 98%) sequence identity with a sequence of SEQ ID NO: 15. In some embodiments, Y comprises a polypeptide of SEQ ID NO: 15.

In some embodiments, Y-B is a chimeric fusion protein. In some embodiments, Y-B is selected from an Fc-TSHR fusion, and an HSA-TSHR fusion.

In some embodiments, Y is covalently linked to B via a linker. In some embodiments, the linker comprises a non-peptidic linking moiety. In some embodiments, Y is fused directly to B. In some embodiments, Y is fused indirectly to B via a spacer domain. In some embodiments, the N-terminal of Y is fused to the C-terminal of B. In some embodiments, the C-terminal of Y is fused to the N-terminal of B. In some embodiments, Y is covalently linked to B via a non-peptidic linking moiety (e.g., a bifunctional linker).

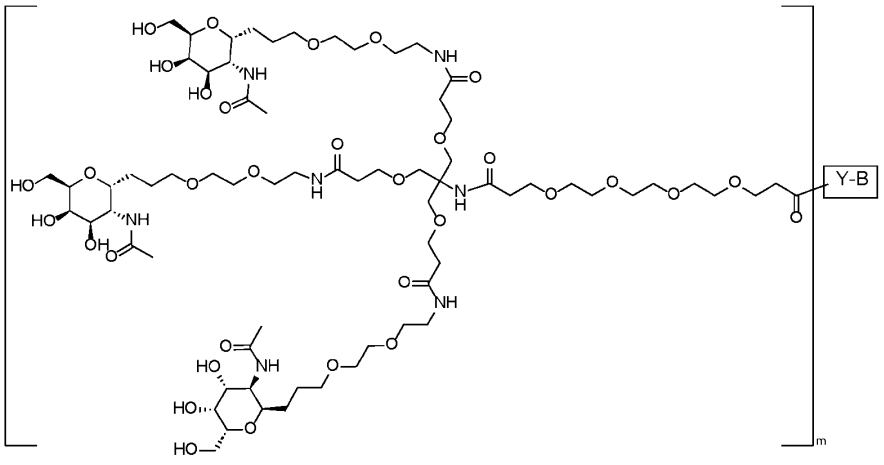
In some embodiments, n is from about 2 to about 10. In some embodiments, n is 3.

In some embodiments, m is 4 to 6.

In some embodiments, Y-B comprises any one of SEQ ID NOs: 1-7. In some embodiments, B comprises any one of SEQ ID NOs: 1-7. In some embodiments, B comprises SEQ ID NO: 6. In some embodiments, Y comprises any one of SEQ ID NOs: 8, 9, 10, 11, 12, 14, and 15. In some embodiments, Y comprises SEQ ID NOs: 15. In some embodiments, B comprises SEQ ID NO: 6 and Y comprises SEQ ID NOs: 15. In some embodiments, B is SEQ ID NO: 6 and Y is SEQ ID NOs: 15.

In some embodiments, L is conjugated to one or more lysine residues of Y or B. In some embodiments, L is conjugated to one or more cysteine residues of Y.

In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula:


HO
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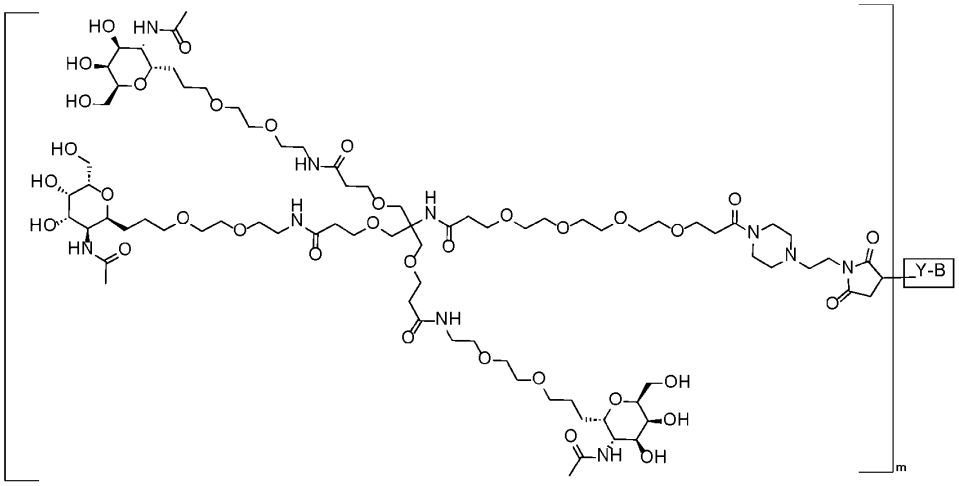


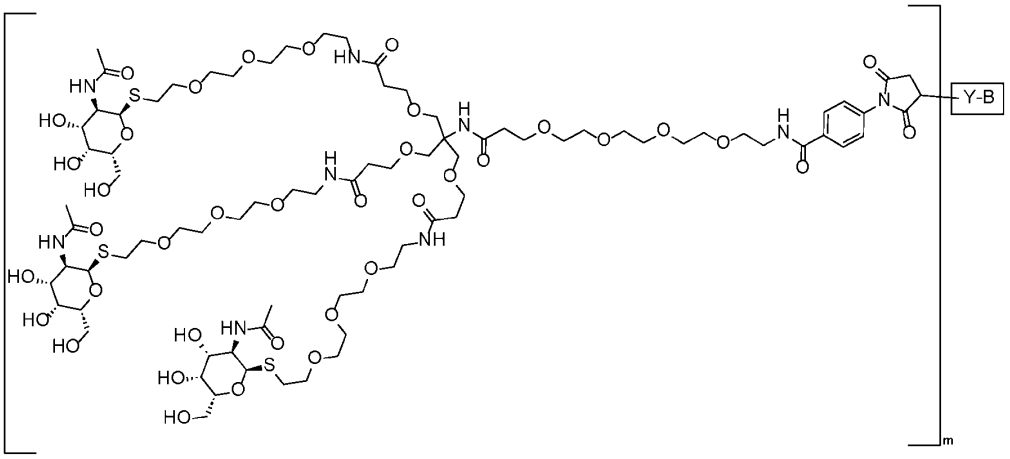
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6.

In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula:



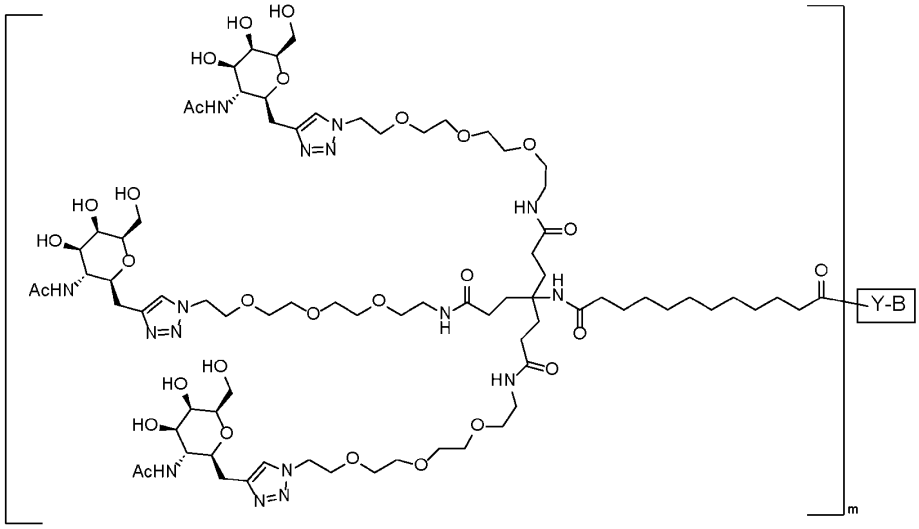
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6.

In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula:



wherein:

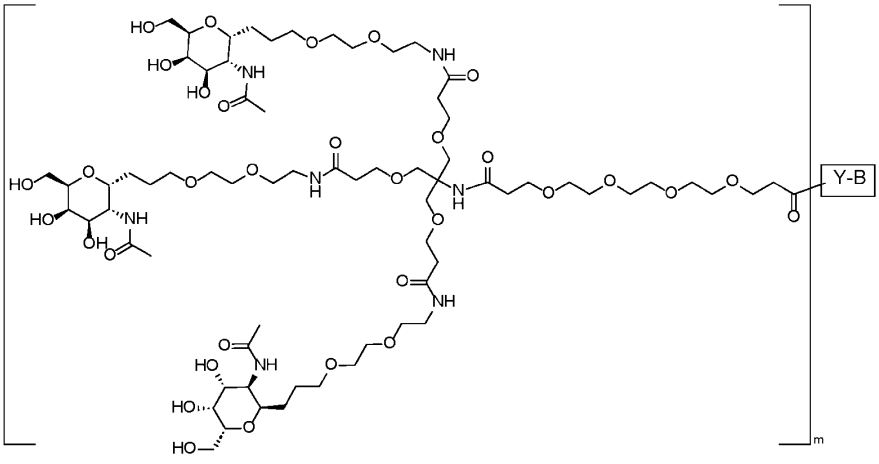
m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6.

In some embodiments, Y is connected to B via a linking sequence. In some embodiments, linking sequence comprises GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula:



wherein:

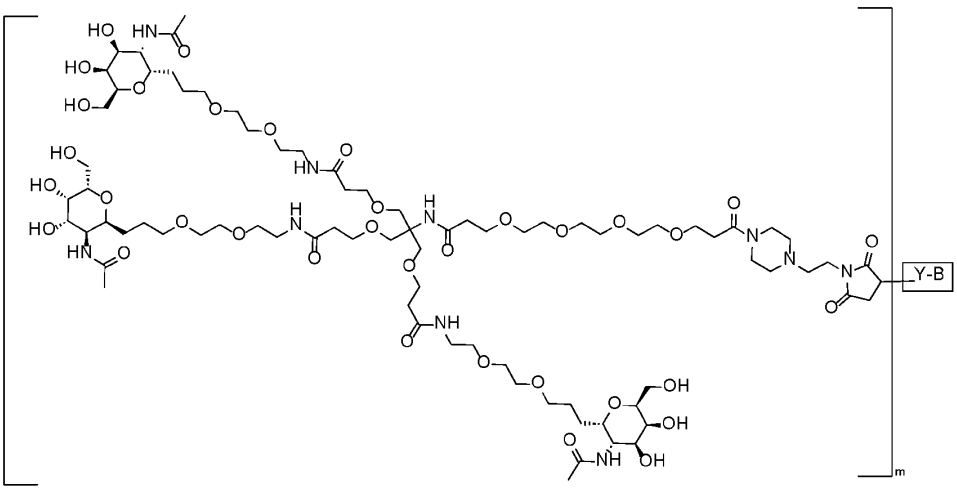
m is 4-6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15 ;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula:



wherein:

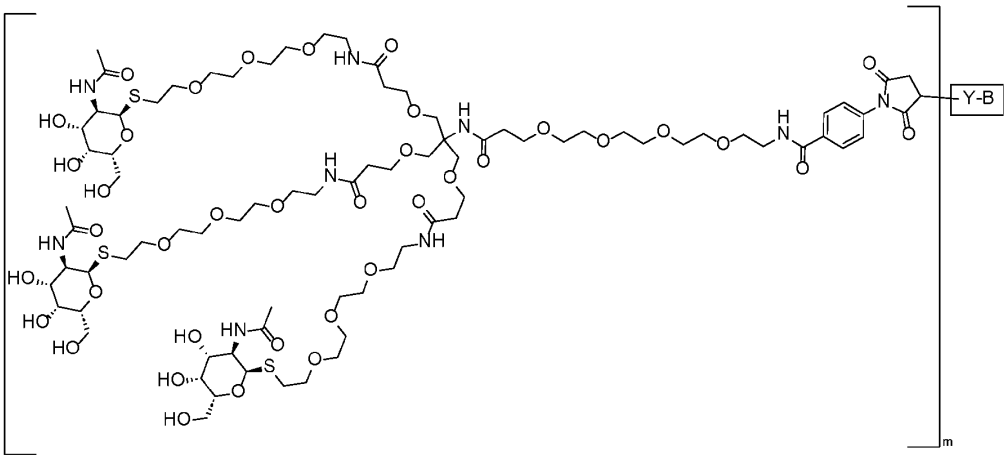
m is 4-6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula:



wherein:

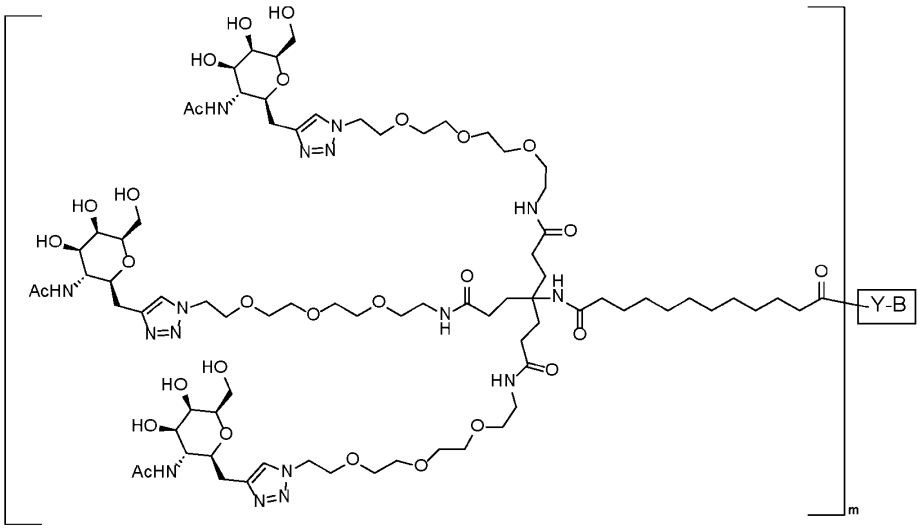
m is 4-6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, provided is a conjugate (i.e., LYTAC), of Formula:



wherein:

m is 4-6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, the conjugate facilitates internalization of anti-TSHR autoantibody into human cells. In some embodiments, the conjugate facilitates accumulation of anti-TSHR autoantibody in an acidic intracellular compartment of human cells. In some embodiments, the conjugate facilitates degradation of anti-TSHR autoantibody by lysosome in the human cells.

In some embodiments, provided is a method of reducing levels of an extracellular anti-TSHR autoantibody in a patient, the method comprising administering an effective amount of a LYTAC or conjugate as described herein. In some embodiments, the administration increases internalization of anti-TSHR autoantibody. In some embodiments, the internalized anti-TSHR autoantibody accumulates into an acidified endosome compartment. In some embodiments, the administration facilitates degradation of the anti-TSHR autoantibody. In some embodiments, the administration results in decrease of greater than 80%

of extracellular anti-TSHR autoantibody level in the patient. In some embodiments, the anti-TSHR autoantibody is M22 or K1-18.

In some embodiments, provided is a method of treating Graves' disease in a patient in need thereof, the method comprising administering to the subject an effective amount of a LYTAC or conjugate as described herein. In some embodiments, the administration increases internalization of anti-TSHR autoantibody. In some embodiments, the internalized anti-TSHR autoantibody accumulates in an acidified endosome compartment. In some embodiments, the administration facilitates degradation of the anti-TSHR autoantibody. In some embodiments, the administration results in decrease of greater than 80% of extracellular anti-TSHR autoantibody level in the patient. In some embodiments, the anti-TSHR autoantibody is M22 or K1-18.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present disclosure will become better understood with regard to the following description and accompanying drawings.

FIGs. 1A-1B show illustrations of LYTAC formats.

FIGs. 2A-C show improved stability and binding of a stabilized form of TSHR over that of the wild-type.

FIGs. 3A-B show the results of a cellular uptake assay of autoantibodies by a TSHR-based LYTAC.

FIG. 4 shows the results of an internalization assay, where LYTACs mediated internalization of an anti-TSHR antibody into an acidified compartment.

FIG. 5 shows results of a cellular uptake assay, where LYTACs with the K250Q mutation performed equivalently to LYTACs that did not contain this mutation.

FIG. 6 shows LYTAC-mediated uptake of a pathogenic anti-TSHR antibody, and comparisons with a non-binding control and in ASGPR1/2 knock-out cells.

FIG. 7 shows that LYTACs do not bind TSH, as measured by BLI.

FIGs. 8A-B show binding kinetics of a LYTAC to two anti-TSHR antibodies.

FIG. 9 shows results of an uptake assay, comparing the effect of LPR on the degree of antibody uptake.

FIGs. 10A-B show results of a degradation assay, where a LYTAC mediated lysosomal degradation of a pathogenic anti-TSHR antibody in a dose-dependent manner.

FIG. 11 shows results of a degradation assay in which LYTACs mediated degradation of a pathogenic anti-TSHR antibody in hepatocytes.

FIG. 12 shows results of a depletion assay in which LYTACs mediated depletion of anti-TSHR autoantibodies from patient serum.

FIGs. 13A-F show results of in vivo experiments where LYTACs mediated clearance of anti-TSHR antibodies.

FIG. 14 shows that a single 10 mg/kg dose of a LYTAC resulted in complete clearance of an anti-TSHR antibody from circulation in vivo.

FIG. 15 shows the results of an active immunization adoptive transfer model, in which LYTACs mediated the clearance of a mixture of anti-TSHR antibodies.

FIG. 16 shows the serum level of biotin-labeled K1-18 hFcRn Tg32 mice after administration of LYTACs to hFcRn Tg32 mice.

DETAILED DESCRIPTION

A lysosome-targeting chimera (LYTAC) is a protein-based therapeutic designed to bind and rapidly clear, via targeted degradation, thyroid stimulating hormone receptor (TSHR) autoantibodies. Structurally, in some embodiments, a fusion protein of a TSHR polypeptide and human serum albumin that is conjugated, using nonspecific lysine conjugation, to a linker-internalizer comprising a non-cleavable linker bound to a stabilized tri-GalNAc derivative. The fusion protein binds to anti-TSHR autoantibodies, while the linker-internalizer promotes rapid systemic clearance mediated by cellular uptake via binding to the liver specific asialoglycoprotein receptor (ASGPR). ASGPR is a heterotrimer with two ASGPR1 and one ASGPR2 subunits. The human ASGPR1 carbohydrate recognition domain shares 79-99% sequence identity across monkey, dog, rabbit, rat and mouse proteins and complete conservation of residues within 5 Å of a bound GalNAc based on a co-crystal structure. No structural information is available for ASGPR2, but the human carbohydrate recognition domain shares 67-96% sequence identity across monkey, dog, rabbit, rat, and mouse proteins. Furthermore, ASGPR-mediated liver distribution of GalNAc-conjugated siRNAs has been demonstrated for rat and monkey (McDougall et al, 2021).

As summarized above, this disclosure provides lysosomal targeting bifunctional molecules (also referred to as conjugates or LYTACs) that target disease causing anti-TSHR autoantibodies for degradation. The lysosomal targeting bifunctional molecules include a ligand moiety that specifically

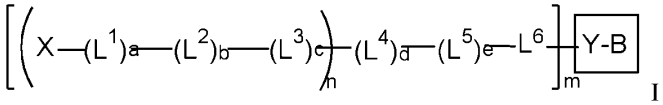
binds to an asialoglycoprotein receptor (ASGPR), and which is linked to a thyroid stimulating hormone receptor (TSHR) polypeptide that specifically binds target anti-TSHR autoantibodies.

1.1 Lysosomal Targeting Bifunctional Molecules (LYTACs)

In some embodiments, a conjugate binds pathogenic anti-TSHR antibodies in circulation and forms a ternary complex with a liver-specific internalizing receptor, ASGPR. After clathrin-mediated endocytosis, the protein complex progresses through the endocytic pathway whereby ASGPR dissociates due to decreasing pH and Ca^{2+} levels and is recycled to the cell surface. A conjugate and the pathogenic antibody continue to the lysosome where they are degraded by lysosomal proteases.

The bifunctional molecules (e.g., conjugates or LYTACs) of this disclosure having a particular configuration of ASGPR binding moieties (X) with a linker of desired valency and/or length can specifically bind with high affinity to both the ASGPR receptor and a target anti-TSHR antibody simultaneously and exhibit high uptake activity of the target autoantibody. The conjugates of this disclosure can provide for sequestering and degrading of a target anti-TSHR autoantibody in the cell's lysosome.

In some embodiments, the lysosomal targeting bifunctional molecule is a conjugate of Formula I:



I

or a prodrug thereof, or a salt thereof, wherein:

n is 1 to 10;

m is 1 to 10;

X is a moiety that binds to asialoglycoprotein receptor (ASGPR);

Y is a carrier polypeptide connected to B;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody;

each L^1 to L^6 is independently a linking moiety which together provide a linear or branched linker between each X and the Y-B complex; and

a, b, c, d, and e are each independently 1, 2, 3, 4, or 5.

In some embodiments, L^6 comprises a residual moiety resulting from the covalent linkage of a thiol-reactive chemoselective ligation group to one or more cysteine residue(s) of Y-B; or L^6 is a residual moiety resulting from the covalent linkage of an amine-reactive chemoselective ligation group to one or more lysine residue(s) of Y-B.

In some embodiments, Y-B is a chimeric fusion protein (i.e., Y-B complex) including a carrier polypeptide and a polypeptide that specifically binds the target anti-TSHR autoantibody. It is to be understood that each X can be independently bonded to Y or B of the Y-B chimeric fusion protein via the linking moiety, e.g., one or more X can be bonded to Y and one or more X can be bonded to B.

Each of the components of the lysosomal targeting bifunctional molecules, configurations of such molecules, and methods of using the same, are now described in greater detail.

In some embodiments, n is from about 2 to about 10. In some embodiments, n is from about 2 to about 9, from about 2 to about 8, from about 2 to about 7, from about 2 to about 6, from about 2 to about 5, from about 2 to about 4. In some embodiments, n is 3. In some embodiments, n is about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In some embodiments, m is about 1 to about 10, about 1 to about 8, about 1 to about 6, about 2 to about 10, about 2 to about 8, about 2 to about 6, about 4 to about 8, about 4 to about 6, about 4 to about 5, about 5 to about 8, or about 5 to about 6. In some embodiments, m is about 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10, or any value between them. In some embodiments, m is about 4 to about 6.

1.1.1 ASGPR Ligand Moieties

An asialoglycoprotein receptor (ASGPR) ligand moiety is a moiety that binds to ASGPR (i.e., also referred to as an ASGPR binding moiety) and, via the ASGPR, facilitates internalization of the bifunctional molecule of which it is a part, plus any bound target molecule, e.g., autoantibody. The ASGPR ligand moieties of this disclosure can be connected via a linker to a polypeptide construct without impacting the specific binding to, or function of, the cell surface ASGPR. The lysosomal targeting bifunctional molecules of this disclosure which include one or more linked ASGPR ligand moieties can utilize the functions of cell surface ASGPRs in a biological system, e.g., for internalization and/or sequestration of autoantibody to the lysosome of a cell, and subsequent lysosomal degradation, e.g., in the methods of this disclosure.

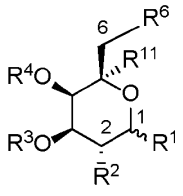
ASGPR, also known as the Ashwell Morell receptor, is a transmembrane glycoprotein receptor found primarily in hepatocytes which mediates the endocytosis and lysosomal degradation of glycoproteins with exposed terminal galactose or N-acetylgalactosamine (GalNAc) residues. ASGPR

cycles between intracellular endosomes and the cell surface. In some embodiments, the ASGPR is *Homo sapiens* asialoglycoprotein receptor 1 (ASGR1) (*see*, *e.g.*, NCBI Reference Sequence: NM_001197216). ASGPR binding compounds and conjugates are described in International Publication WO2023/288033 or WO2022/142377 the disclosure of which are incorporated by reference herein in their entirety.

In some embodiments, the ASGPR binding moiety (X) includes an amino sugar ring analog of galactose (e.g., N-acetylgalactosamine, or analogs thereof) that is connected to a linker scaffold via an optional linking moiety at the 1-, 2- or 6-position of the sugar ring analog. In some embodiments, the linking moiety includes an oxygen, sulfur, nitrogen or carbon atom connected at the 1-position of the ring. In some embodiments, the linking moiety includes an oxygen, sulfur, nitrogen, or carbon atom connected at the 2-position of the ring. In some embodiments, the linking moiety includes an oxygen, sulfur, nitrogen, or carbon atom connected at the 1-position of the ring. In some embodiments, the linking moiety connected at the 1-, 2-, or 6-position of the ring includes an optionally substituted aryl or heteroaryl group. In some embodiments, the amino sugar ring analog of galactose has a bicyclic structure.

In some embodiments, the ASGPR ligand moieties (e.g., each X, optionally with the linker or a portion thereof, prior to conjugation) of the bifunctional molecule specifically bind to ASGPR with an affinity (K_d) of 300 nM or less, such as 100 nM or less, 30 nM or less, 10 nM or less, 3 nM or less, or 1 nM or less. The terms "binds," "binds to," "specifically binds," or "specifically binds to" in this context are used interchangeably.

In some embodiments of the conjugates described herein, X is an asialoglycoprotein receptor (ASGPR) binding moiety of Formula II:



II

wherein:

R^1 is selected from -Z^1-*, -H, -OH, optionally substituted (C_1-C_6)alkyl, -OCH_3,-OCH_2CH=CH, optionally substituted -S-(C_1-C_6)alkyl, optionally substituted aryl, optionally substituted heteroaryl, optionally substituted -S-aryl, and optionally substituted -S-heteroaryl;

R^2 is selected from -Z^1-*, -$NHCOCH_3$, -$NHCOCF_3$, -$NHCOCH_2CF_3$, -OH, -NHR, and optionally substituted triazole;

R^6 is selected from -Z^1-*, -OH, -OR, optionally substituted (C_1-C_6)alkyl, -OC(O)R, -C(O)NHR, -$NR^{xx}R^{yy}$, optionally substituted aryl, optionally substituted heteroaryl, -NHCOR, and -NRCOR;

each R is independently optionally substituted (C_1-C_6)alkyl, optionally substituted aryl, or optionally substituted heteroaryl;

R^{xx} and R^{yy} are independently H, optionally substituted (C_1-C_6)alkyl, or R^{xx} and R^{yy} can cyclize to form an optionally substituted heterocyclyl;

wherein one of R^1, R^2, and R^6 is -Z^1-*, and " * " represents a point of connection of Z^1 to the linker (L);

R^3 and R^4 are each independently H, or a promoiety, or R^3 and R^4 are cyclically linked to form a promoiety;

R^{11} is H, or a bridging moiety that connects the 5-position carbon to the 1-position carbon of the ring;

Z^1 is a linking moiety selected from -Z^{11}-, -Z^{11}-A^1-*, -A^2-, -NR^{21}CO-*, - $CONR^{21}$-*, -$NR^{21}SO_2$*-, -SO_2NR^{21}-*, -NR^{21}C(=O)NR^{21}-, and -NR^{21}C(=S)NR^{21}-;

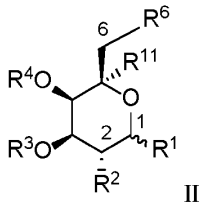
-Z^{11}- is -O-, -S-, -N(R^{21})-, or -C(R^{22})$_2$;

-A^1- and -A^2- are optionally substituted heterocyclylene, optionally substituted arylene, or optionally substituted heteroarylene;

each R^{21} is independently selected from H, optionally substituted (C_1-C_6)alkyl, -COR, and optionally substituted heteroaryl; and

each R^{22} is independently selected from H, halogen, and optionally substituted (C_1-C_6)alkyl.

In some embodiments, the lysosomal targeting bifunctional molecules of this disclosure (e.g., of Formula I) can include an ASGPR ligand moiety of Formula II:



II

wherein:

R^1 is selected from -Z^1-*, -H, -OH, -CH_3, -OCH_3, and -OCH_2CH=CH;

R^2 is selected from -Z^1-*, -$NHCOCH_3$, -$NHCOCF_3$, -$NHCOCH_2CF_3$, -OH, and optionally substituted triazole;

R^6 is selected from -Z^1-*, -OH, -OC(O)R, -C(O)NHR, and optionally substituted triazole, where R is optionally substituted (C_1-C_6)alkyl or optionally substituted aryl;

wherein one of R^1, R^2, and R^6 is -Z^1-*, and " * " represents a point of connection of Z^1 to the linker (L);

R^3 and R^4 are each independently H, or a promoiety, or R^3 and R^4 are cyclically linked to form a promoiety;

R^{11} is H, or a bridging moiety that connects the 5-position carbon to the 1-position carbon of the ring;

Z^1 is a linking moiety selected from -Z^{11}-, -Z^{11}-A^1-*, -A^2-, -$NR^{21}CO$-*, - $CONR^{21}$-*, -$NR^{21}SO_2$-*, -SO_2NR^{21}-*, -$NR^{21}C(=O)NR^{21}$-, and -$NR^{21}C(=S)NR^{21}$-;

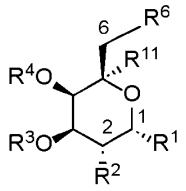
-Z^{11}- is -O-, -S-, -$N(R^{21})$-, or -$C(R^{22})_2$;

-A^1- and -A^2- are optionally substituted arylene or optionally substituted heteroarylene;

each R^{21} is independently selected from H, and optionally substituted (C_1-C_6)alkyl; and

each R^{22} is independently selected from H, halogen (e.g., F) and optionally substituted (C_1-C_6)alkyl.

In some embodiments, X is represented by Formula a-II:



a-II.

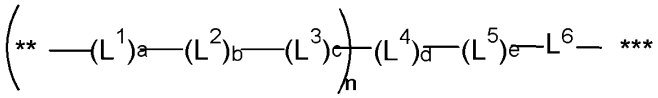
In some embodiments, R^1 is -Z^1-*, -H, or (C_1-C_6)alkyl. In some embodiments, R^1 is -Z^1-*, -H, or n-propyl.

In some embodiments, R^2 is -Z^1-* or -$NHCOCH_3$.

In some embodiments, R^3 and R^4 are each -H.

In some embodiments, L comprises of 10 to 60 consecutive branched or linear chain atoms.

In some embodiments, L is of Formula:



wherein:

n is 1, 2, or 3;

each L^1 to L^6 is independently a linking moiety which together provide a linear or branched linker between Z^1 and Y;

a, b, c, d, and e are each independently 1, 2, 3, 4, or 5;

** represents the point of attachment of X via Z^1 to L^1; and

*** represents the point of attachment to Y.

In some embodiments, each L^1 to L^5 independently comprises one or more linking moieties independently selected from -C_{1-20}-alkylene-, -NHC(O)-C_{1-6}-alkylene-, -C(O)NH-C_{1-6}-alkylene-, -NHC$_{1-6}$-alkylene-, -NHC(O)NH-C_{1-6}-alkylene-, -NHC(S)NH-C_{1-6}-alkylene-, -C_{1-6}-alkylene-NHC(O)-, -C_{1-6}-alkylene-C(O)NH-, -C_{1-6}-alkylene-NH-, -C_{1-6}-alkylene-NHC(O)NH-, -C_{1-6}-alkylene-NHC(S)NH-, -$O(CH_2)_p$-, -$(OCH_2CH_2)_p$-, -NHC(O)-, -C(O)NH-, -$NHS(O)_2$-, -$S(O)_2NH$-, -C(O)-, -$S(O)_2$-, -O-, -S-, monocyclic heteroaryl, monocyclic aryl, monocyclic heterocycle, monocyclic carbocycle, amino acid residue, -NH-, and -NCH_3-; wherein each L^1 to L^5 is independently optionally substituted with one to five halo;

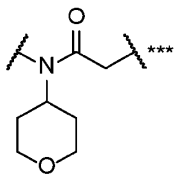
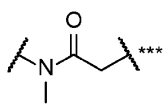
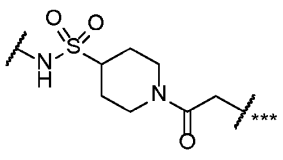
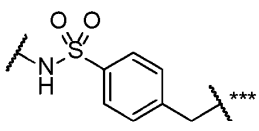
each p is independently1 to 50;

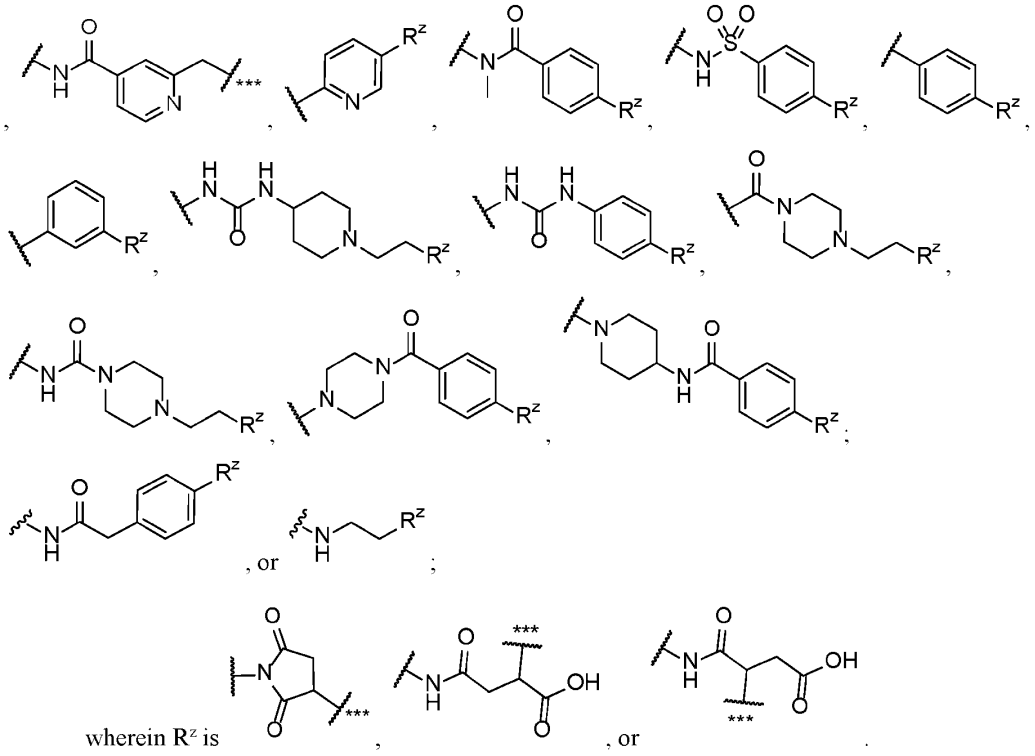
L^6 is a linking group comprising one or more linking moieties independently selected from -C_{1-20}-alkylene-, -NR^{16}C(O)-C_{1-6}-alkylene-, -C(O)NR^{16}-C_{1-6}-alkylene-, -NR^{16}-C_{1-6}-alkylene-, -NR^{16}C(O)NR^{16}-C_{1-6}-alkylene-, -NR^{16}C(S)NR^{16}-C_{1-6}-alkylene-, -C_{1-6}-alkylene-NR^{16}C(O)-, -C_{1-6}-alkylene-C(O)NR^{16}-, -C_{1-6}-alkylene-NR^{16}-, -C_{1-6}-alkylene-NR^{16}C(O)N R^{16}-, -C_{1-6}-alkylene-NR^{16}C(S)NR^{16}-, -$O(CH_2)_p$-, -$(OCH_2CH_2)_p$-, -NR^{16}C(O)-, -C(O)NR^{16}-, -$NHS(O)_2$-, -$S(O)_2NH$-, -C(O)-, -$S(O)_2$-, -O-, -S-, monocyclic heteroaryl, monocyclic aryl, monocyclic heterocycle, amino acid residue, or -NR^{16}-; and

each R^{16} is independently -H, optionally substituted (C_1-C_6)alkyl, optionally substituted aryl, optionally substituted monocyclic heteroaryl or monocyclic heteroaryl.

In some embodiments, each L^1 to L^5 is independently selected from -C_{1-20}-alkylene-, -NHC(O)-C_{1-6}-alkylene-, -C(O)NH-C_{1-6}-alkylene-, -NH-C_{1-6}-alkylene-, -NHC(O)NH-C_{1-6}-alkylene-, -NHC(S)NH-C_{1-6}-alkylene-, -C_{1-6}-alkylene-NHC(O)-, -C_{1-6}-alkylene-C( )NH-, -C_{1-6}-alkylene-NH-, -C_{1-6}-alkylene-NHC(O)NH-, -C_{1-6}-alkylene-NHC(S)NH-, -$O(CH_2)_p$-, -$(OCH_2CH_2)_p$-, -NHC(O)-, -C(O)NH-, -$NHS(O)_2$-, -$S(O)_2NH$-, -C(O)-, -$S(O)_2$-, -O-, -S-, monocyclic heteroaryl, monocyclic aryl, monocyclic heterocycle, monocyclic carbocycle, amino acid residue, -NH-, and -NCH_3-; wherein each L^1 to L^5 is independently optionally substituted with one to five halo;

each p is independently1 to 50; and

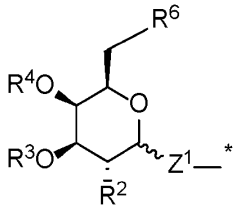
L^6 is , , , 



wherein R^z is

1-linked ASGPR ligand moieties

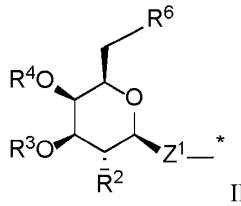
In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by Formula IIa:



IIa

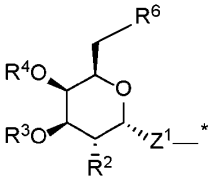
wherein R^2, R^3, R^4, R^6 and Z^1 are as defined herein. In some embodiments of Formula IIa, R^6 is selected from -OH, -OC(O)R, and -C(O)NHR; and R^2 is selected from $-NHCOCH_3$, $-NHCOCF_3$, and $-NHCOCH_2CF_3$.

In some embodiments of Formula II, Z^1 is in a beta configuration, and can be described by Formula IIa-1:



IIa-1.

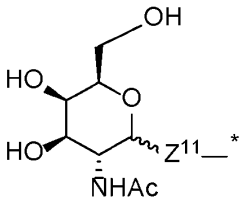
In some embodiments of Formula II, Z^1 is in an alpha configuration, and can be described by Formula IIa-2:



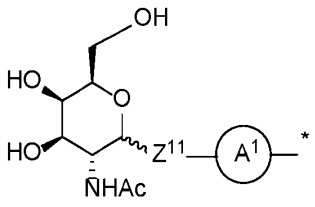
IIa-2.

In some embodiments of Formula IIa, IIa-1, or IIa-2, Z^1 is $-Z^{11}-A^1-$, wherein A^1- is optionally substituted arylene or optionally substituted heteroarylene. In some embodiments, A^1 is an optionally substituted heteroarylene. In some embodiments, the heteroarylene is a 5 or 6-membered heteroarylene. In some embodiments, the heteroarylene is a 5-membered heteroarylene. In some embodiments, the 5-membered heteroarylene is a triazole. In some embodiments, the triazole is a 1,2,3-triazole moiety.

In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by Formula IIIa or IIIb:



IIIa



IIIb

wherein:

$-Z^{11}-$ is -O-, -S-, $-N(R^{21})-$, or $-C(R^{22})_2-$, where each R^{22} is independently selected from H, halogen (e.g., F) and optionally substituted (C_1-C_6)alkyl, and R^{21} is H or optionally substituted (C_1-C_6)alkyl; and

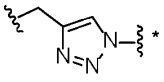
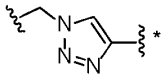
$-A^1-$ is arylene, substituted arylene, heteroarylene, or substituted heteroarylene.

In some embodiments, $-A^1-$ is arylene or heteroarylene; wherein each is independently optionally substituted with one to three halo, C_{1-3}alkyl, C_{1-3}haloalkyl, C_{1-3}alkoxy, or C_{1-3}haloalkoxy.

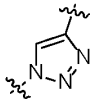
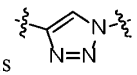
In some embodiments of Formula IIIa or IIIb, Z^{11} is -S-.

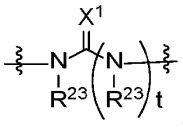
In some embodiments, Z^{11} is $-C(R^{22})_2-$. In some embodiments, Z^{11} is $-CH_2-$.

In some embodiments, Z^{11} is $-C(R^{22})_2$, where at least one R^{22} is H. In some embodiments, both R^{22} are H. In some embodiments Z^{11} is -O-. In some embodiments, Z^{11} is -S-. In some embodiments cases, Z^{11} is $-N(R^{21})$, where R^{21} is H or $C_{1\text{-}3}$alkyl.

In some embodiments, $-A^1-$ is triazole. In some embodiments, Z^1 is $-C(R^{22})_2$-triazole-. In some embodiments, Z^1 is:  . In some embodiments, Z^1 is:  .

In some embodiments of Formula IIa, IIa-1, or IIa-2, Z^1 is Z^{11}. In some embodiments, Z^{11} is $-C(R^{22})_2$. In some embodiments, at least one R^{22} is H. In some embodiments, both R^{22} are H, and Z^{11} is $-CH_2-$. In some embodiments Z^{11} is -O-. In some embodiments, Z^{11} is -S-. In certain other cases, Z^{11} is $-N(R^{21})$, where R^{21} is H or $C_{1\text{-}3}$alkyl.

In some embodiments of Formula IIa, IIa-1, or IIa-2, Z^1 is monocyclic 5 or 6-membered heteroaryl or aryl. In some embodiments, Z^1 is  . In some embodiments, Z^1 is  .

In some embodiments of Formula IIa, IIa-1, or IIa-2, Z^1 is selected from -O-, -S-, $-C(R^{22})_2-$, $-NR^{21}-$, $-CONR^{21}-$, and  ;

wherein:

X^1 is O or S;

t is 0 or 1;

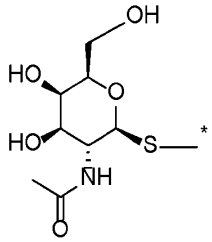
R^{21} and each R^{23} is independently selected from H, and optionally substituted $(C_1\text{-}C_6)$alkyl (e.g., $C_{(1\text{-}3)}$-alkyl, such as methyl); and

each R^{22} is independently selected from H, halogen (e.g., F) and optionally substituted $(C_1\text{-}C_6)$alkyl.

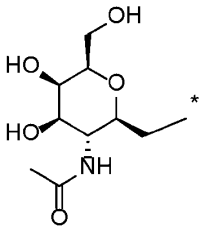
In some embodiments of Formula IIa, IIa-1, or IIa-2, Z^1 is optionally substituted $(C_1\text{-}C_6)$alkyl. In some cases of Z^1 the alkyl is methyl. In some cases of Z^1, the alkyl is ethyl. In some cases of Z^1, the

alkyl is propyl. In some cases of Z^1, the alkyl is butyl. In some cases of Z^1, the alkyl is pentyl. In some cases of Z^1, the alkyl is hexyl.

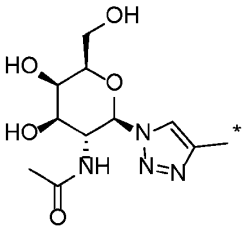
In some embodiments, the ASGPR binding moiety (X) of Formula IIa-1 is selected from one of the following structures:



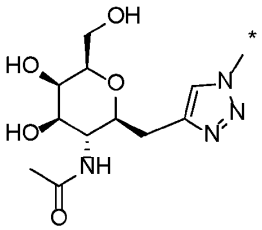
X^A-1,



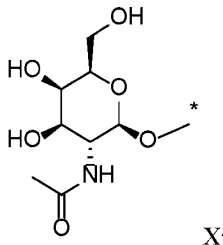
X^A-2,



X^A-3,

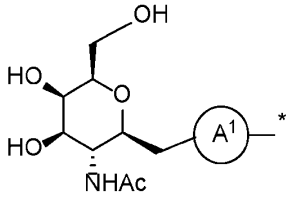


X^A-4, and



X^A-5.

In some embodiments of Formula IIa-2, Z^1 is in a beta configuration and X is of Formula IIIb-2:



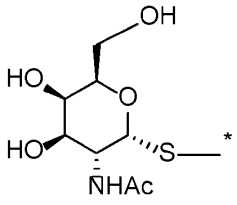
IIIb-2

wherein: -A^1- is arylene, substituted arylene, heteroarylene, or substituted heteroarylene.

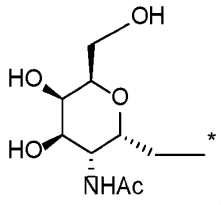
In some embodiments of Formula IIIb-2, A^1 is a triazole. In some embodiments of Formula IIIb-2, X is of Formula X^A-4.

In some embodiments of Formula IIa-1, Z^1 is in a beta configuration at the 1-position carbon of the galactosamine ring. In some embodiments of Formula IIa-1, Z^1 is S, and each X is of Formula X^A-1. In some embodiments of Formula IIa-1, each X is of formula X^A-2. In some embodiments of Formula IIa-1, each X is of Formula X^A-3. In some embodiments of Formula IIa-1, each X is of Formula X^A-4. In some embodiments of Formula IIa-1, each X is of Formula X^A-5.

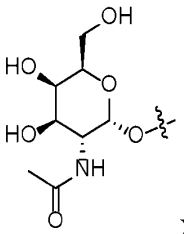
In some embodiments, the compound of Formula IIa-2 is selected from one of the following structures:



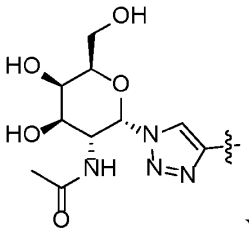
X^B-1,



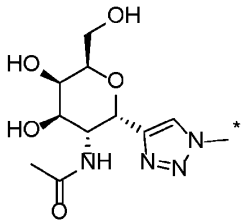
X^B-2,



X^B-3,

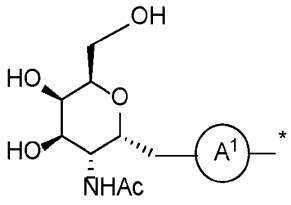


X^B-4 and



X^B-5.

In some embodiments of Formula IIa-2, Z^1 is in an alpha configuration and X is of Formula IIIb-1:

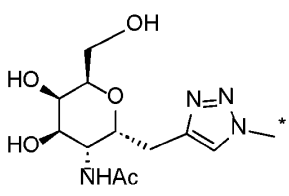


IIIb-1

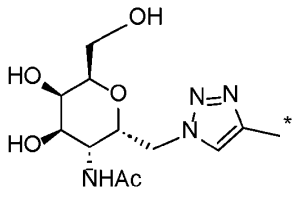
wherein -A^1- is arylene, substituted arylene, heteroarylene, or substituted heteroarylene.

In some embodiments of Formula IIIb-1, A^1 is an optionally substituted heteroarylene. In some embodiments, the heteroarylene is a 5 or 6-membered heteroarylene. In some embodiments, the heteroarylene is a 5-membered heteroarylene. In some embodiments, the 5-membered heteroarylene is a triazole. In some embodiments, the triazole is a 1,2,3-triazole moiety. In some embodiments, each is independently optionally substituted with one to three halo, $C_{1\text{-}3}$alkyl, $C_{1\text{-}3}$haloalkyl, $C_{1\text{-}3}$alkoxy, or $C_{1\text{-}3}$haloalkoxy.

In some embodiments, the X of Formula IIIb-1 is selected from one of the following structures:

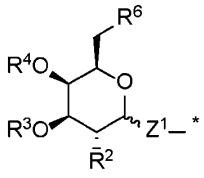


X^C-1



X^C-2

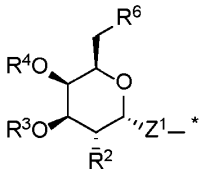
In some embodiments of Formula IIa:



IIa

R^6 is -OH, R^2 is $-NHC(O)CH_3$, R^3 is H or $-C(O)CH(CH_3)_2$, R^4 is H, and Z^1 is -O-, -S-, $-CH_2-$, -NH-, or a 1,2,3-triazolyl.

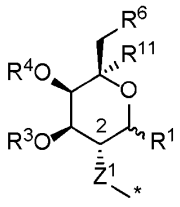
In some embodiments, Z^1 is in the alpha configuration such that the ASGPR binding moiety is derived from Formula IIa-2:



IIa-2.

2-linked ASGPR ligand moieties

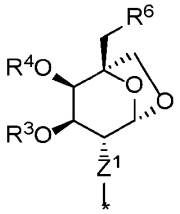
In some embodiments, the ASGPR binding moiety (X) is linked via the 2-postion of the sugar analog. In some embodiments, the ASGPR binding moiety (X) has a reduced ring carbon at the 1-position relative to a galactosamine derived sugar. In some embodiments, the ASGPR binding moiety (X) of the bifunctional molecules of this disclosure is described by Formula IIb:



IIb

wherein R^1, R^3, R^4, R^6, R^{11}, and Z^1 are as defined herein.

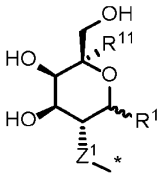
In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure are described by Formula IIb':



IIb'

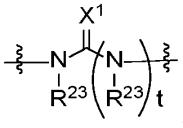
wherein R^3-R^4, R^6, and Z^1 are as defined herein.

In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure are described by Formula IVa:

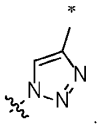
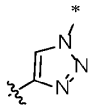


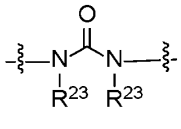
IVa

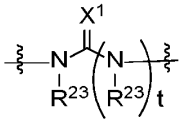
wherein R^1, R^{11}, and Z^1 are as defined herein.

In some embodiments of Formula IIb, IIb', or IVa, Z^1 is selected from optionally substituted -$(C(R^{22})_2)_q$-heteroarylene, and , wherein t is 0 or 1.

In some embodiments of Formula IIb, IIb' or IVa, Z^1 is optionally substituted -$(C(R^{22})_2)_q$-triazole, wherein q is 0 or 1. In some embodiments of Formula IIb, IIb' or IVa, Z^1 is -$(C(R^{22})_2)_q$-1,2,3-triazolyl, wherein q is 0 or 1.

In some embodiments of Formula IIb, IIb' or IVa, Z^1 is . In some embodiments, Z^1 is .

In some embodiments of Formula IIb, IIb' or IVa, Z^1 is , wherein R^{23} is H, or $C_{(1-3)}$-alkyl. In some embodiments of Formula IIb, IIb' or IVa, Z^1 is -$NR^{23}CO$-, wherein R^{23} is H or $C_{(1-3)}$-alkyl.

In some embodiments of Formula IIb, IIb' or IVa, Z^1 is selected from -O-, -S-, -$C(R^{22})_2$-, -NR^{21}-, -$CONR^{21}$-, and ;

wherein:

X^1 is O or S;

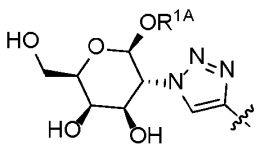
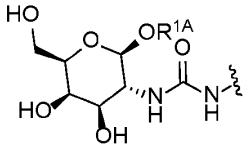
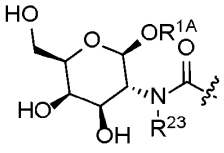
t is 0 or 1;

R^{21} and each R^{23} is independently selected from H, and optionally substituted C_1-$_6$alkyl (e.g., $C_{(1-3)}$-alkyl, such as methyl); and

each R^{22} is independently selected from H, halogen (e.g., F) and optionally substituted C_1-$_6$alkyl.

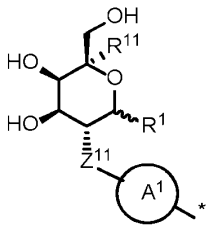
In some embodiments, each is independently optionally substituted with one to three halo, C_{1-3}alkyl, C_{1-3}haloalkyl, C_{1-3}alkoxy, or C_{1-3}haloalkoxy.

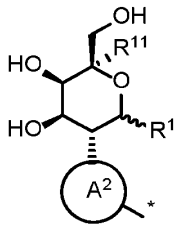
In some embodiments, each X is independently selected from:

, , and 

wherein R^{1A} is independently H or (C_1-$_3$)alkyl.

In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by Formula IVb or IVc:


IVb


IVc

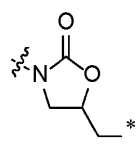
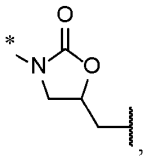
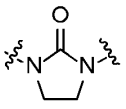
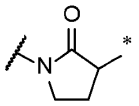
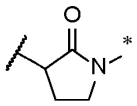
wherein:

$-Z^{11}-$ is -O-, -S-, $-N(R^{21})-$, or $-C(R^{22})_2$;

$-A^1-$ and $-A^2-$ are optionally substituted arylene or optionally substituted heteroarylene;

each R^{21} is independently selected from H, and optionally substituted $(C_1\text{-}C_6)$alkyl; and

each R^{22} is independently selected from H, halogen (e.g., F) and optionally substituted $(C_1\text{-}C_6)$alkyl. In some embodiments of Formula IVb or IVc, R^1 is H.

In some embodiments, $-Z^1-*$ or $-Z^1-L^1-$ comprises , , , , or .

In some embodiments, Z^{11} is $-C(R^{22})_2$. In some embodiments, at least one R^{22} is H. In some embodiments, both R^{22} are H. In some embodiments, Z^{11} is -O-. In some embodiments, Z^{11} is -S-. In some embodiments, Z^{11} is $-N(R^{21})$, where R^{21} is H or $(C_1\text{-}C_3)$alkyl.

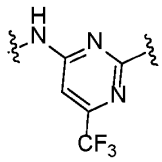
In some embodiments of Formula IVb, IVc, IVb-1, or IVc-1, $-A^1-$ and $-A^2-$ are each independently an optionally substituted heteroarylene. In some embodiments, the heteroarylene is a 5 or 6-membered heteroarylene. In some embodiments, the heteroarylene is a 5-membered heteroarylene. In some embodiments, the heteroarylene is a 6-membered heteroarylene.

In some embodiments of Formula IVb, or IVb-1, the A^1 ring is a 5-membered heteroarylene selected from triazole, thiadiazole, thiophene, oxazole, isoxazole, isothiazole, thiazole, oxadiazole, and furan. In some embodiments, the A^1 ring is a 6-membered heteroarylene selected from pyridine, pyrimidine, pyridazine, pyrazine, and triazine. In some embodiments, the A^1 ring is triazole. In some embodiments, the A^1 ring is pyridine. In some embodiments, the A^1 ring is pyrimidine. In some

embodiments, the A^1 ring is thiadiazole. In some embodiments, the A^1 ring is a 5 or 6-membered arylene or heteroarylene that is further substituted with one or more substituents. In some cases, the A^1 ring is further substituted with one or more substituents selected from halogen, $(C_1$-$C_6)$alkyl and substituted $(C_1$-$C_6)$alkyl (e.g., CF_3).

In some embodiments, the A^2 ring is a 5-membered heteroarylene selected from triazole, thiadiazole, thiophene, oxazole, isoxazole, isothiazole, thiazole, oxadiazole, and furan. In some embodiments, the A^2 ring is a 6-membered heteroarylene selected from pyridine, pyrimidine, pyridazine, pyrazine, and triazine. In some embodiments, the A^2 ring is triazole. In some embodiments, the A^2 ring is pyridine. In some embodiments, the A^2 ring is pyrimidine. In some embodiments, the A^2 ring is thiadiazole. In some embodiments, the A^2 ring is a 5 or 6-membered arylene or heteroarylene that is further substituted with one or more substituents. In some cases, the A^2 ring is further substituted with one or more substituents selected from halogen, $(C_1$-$C_6)$alkyl and substituted $(C_1$-$C_6)$alkyl (e.g., CF_3).

In some embodiments, $-Z^{11}$-A^1- is a monocyclic 5 or 6-memebered heteroarylene of one of the following structures: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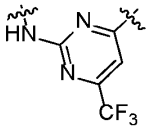
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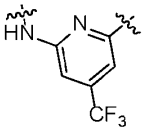
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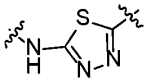
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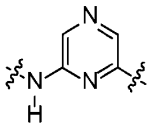
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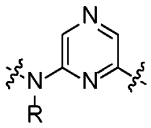
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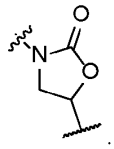
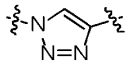
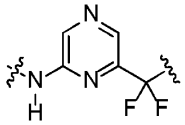

or .

In some embodiments, $-A^2$- is a monocyclic 5 or 6-membered heteroarylene of the structure:

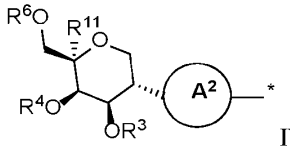

.

In some embodiments, $-Z^{11}$-A^1-L^1 is 
.

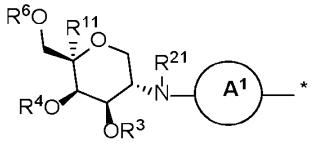
It is understood that a variety of substituents can be utilized to connect a particular $-Z^{11}$-A^1- group to an adjacent linker.

In some embodiments of Formula IIb, or IVa-IVc, R^1 is H, such that the compound of Formula IIb, or IVa-IVc has no non-hydrogen substituents at the 1-position of the sugar ring.

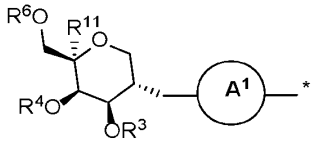
In some embodiments, the compound of Formula IIb is of any one of Formula IVd-IVg:



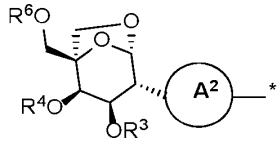
IVd



IVe



IVf, and



IVg

wherein the A^1 and A^2 rings, R^6, R^4, R^3, R^{11}, and R^{21} are as defined herein.

In some embodiments of any one of Formula IVd-IVg, the A^1 ring is a 5 or 6-membered arylene or heteroarylene. In some embodiments, the A^1 ring is a 5-membered heteroarylene selected from triazole, thiadiazole, thiophene, oxazole, isoxazole, isothiazole, thiazole, oxadiazole, and furan. In some embodiments, the A^1 ring is a 6-membered heteroarylene selected from pyridine, pyrimidine, pyridazine, pyrazine, and triazine. In some embodiments, the A^1 ring is triazole. In some embodiments, the A^1 ring is pyridine. In some embodiments, the A^1 ring is pyrimidine. In some embodiments, the A^1 ring is thiadiazole. In some embodiments, the A^1 ring is a 5 or 6-membered arylene or heteroarylene that is further substituted with one or more substituents. In some cases, the A^1 ring is further substituted with one or more substituents selected from halogen, $(C_1\text{-}C_6)$alkyl and substituted $(C_1\text{-}C_6)$alkyl (e.g., CF_3).

In some embodiments of any one of Formula IVd-IVg, the A^2 ring is a 5 or 6-membered arylene or heteroarylene. In some embodiments, the A^2 ring is a 5-membered heteroarylene selected from triazole, thiadiazole, thiophene, oxazole, isoxazole, isothiazole, thiazole, oxadiazole, and furan. In some embodiments, the A^2 ring is a 6-membered heteroarylene selected from pyridine, pyrimidine, pyridazine, pyrazine, and triazine. In some embodiments, the A^2 ring is triazole. In some embodiments, the A^2 ring is pyridine. In some embodiments, the A^2 ring is pyrimidine. In some embodiments, the A^2 ring is thiadiazole. In some embodiments, the A^2 ring is a 5 or 6-membered arylene or heteroarylene that is further substituted with one or more substituents. In some cases, the A^2 ring is further substituted with one or more substituents selected from halogen, $(C_1\text{-}C_6)$alkyl and substituted $(C_1\text{-}C_6)$alkyl (e.g., CF_3).

In some embodiments of any one of Formula IVd-IVg, the A^1 or A^2 ring is absent.

In some embodiments of any one of Formula IVd-IVg, the A^1 or A^2 ring is phenylene or substituted phenylene.

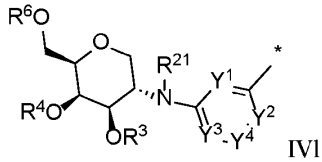
In some embodiments of Formula IVd, the A^2 ring is a 5 or 6-membered heteroarylene. In some embodiments of Formula IVd, the A^2 ring is a 5-membered heteroarylene. In some embodiments of Formula IVd, the A^2 ring is triazole. In some embodiments of Formula IVd, the A^2 ring is absent.

In some embodiments of Formula IVe, the A^1 ring is a 5 or 6-membered heteroarylene and R^{21} is H. In some embodiments of Formula IVe, the A ring is triazole. In some embodiments of Formula IVe, the A^1 ring is pyridine. In some embodiments of Formula IVe, the A^1 ring is pyrimidine. In some embodiments of Formula IVe, the A^1 ring is thiadiazole. In some embodiments of Formula IVe, the A^1 ring is absent and R^{21} is H or optionally substituted acyl. In some cases, R^{21} is $-COCH_3$. In some cases, R^{21} is H.

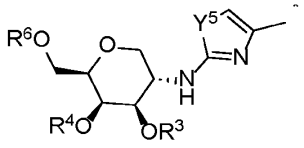
In some embodiments of Formula IVf, the A^1 ring is a 5 or 6-membered heteroarylene. In some embodiments of Formula IVf, the A^1 ring is a 5-membered heteroarylene. In some embodiments of Formula IVf, the A^1 ring is triazole. In some embodiments of Formula IVf, the A^1 ring is absent.

In some embodiments of Formula IVg, the A^2 ring is a 5 or 6-membered heteroarylene. In some embodiments of Formula IVg, the A^2 ring is a 5-membered heteroarylene. In some embodiments of Formula IVg, the A^2 ring is triazole. In some embodiments of Formula IVg, the A^2 ring is absent.

In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by any one of Formula IVl-IVm:



IVl



IVm

wherein:

R^6, R^4, R^3, and R^{21} are as defined herein;

Y^1-Y^3 are each independently N or CR^{25};

Y^4 is N or CR^{24};

Y^5 is S, O, or NH; and

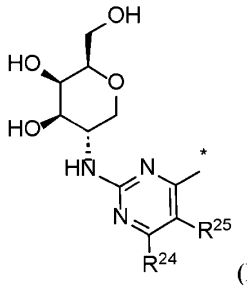
R^{24} and R^{25} are each independently selected from H, optionally substituted $C_{(1-6)}$-alkyl, optionally substituted fluoroalkyl, and halogen.

In some embodiments of Formula IVi at least one of Y^1 to Y^3 is N. In some cases, at least two of Y^1 to Y^3 are N. In some embodiments, Y^1 and Y^3 are N and Y^2 is CR^{25}. In some embodiments, Y^1 and Y^2 are N and Y^3 is CR^{25}. In some embodiments, Y^1 and Y^2 are CR^{25} and Y^3 is N.

In some embodiments of any one of formulae IVd-IVk R^6 is H.

In some embodiments of any one of Formula IVd-IVk, R^4 and R^3 are each H. In some embodiments, at least one of R^4-R^3 is a promoiety. In some embodiments, R^4 and R^3 are cyclically linked to form a promoiety (e.g., as described herein).

In some embodiments, X is of Formula IVi-1:

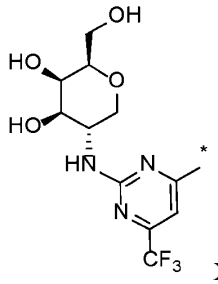


(IVi-1)

wherein R^{24} and R^{25} are independently selected from H, halogen, C_{1-6}-alkyl and substituted C_{1-6}-alkyl (e.g., CF_3).

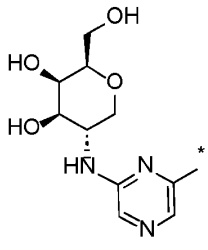
In some embodiments of Formula IVi - IVi-1, R^{25} is H. In some embodiments, R^{25} is C_{1-3}-alkyl, or $C_{(1-3)}$-fluoroalkyl. In some cases, the fluoroalkyl is -CF_3. In some embodiments of Formula IVi or IVi-1, R^{24} is H. In some embodiments, R^{24} is C_{1-3}-alkyl, or C_{1-3}-fluoroalkyl. In some cases, the fluoroalkyl is -CF_3.

In some embodiments, X is of Formula X^{D1}:



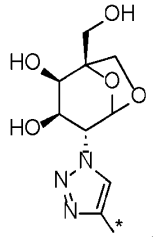
X^{D1}.

In some embodiments, X is of Formula X^{D2}:



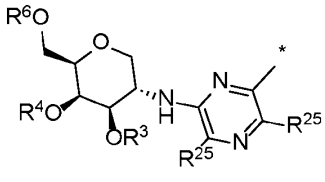
X^{D2}.

In some embodiments, X is of Formula X^E:



X^E.

In some embodiments, X is of Formula IVl-1:



IVl-1

wherein:

R^6, R^4, R^3, and R^{21} are as defined herein;

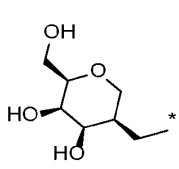
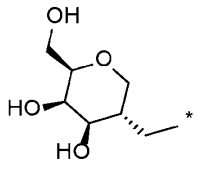
Y^1-Y^4 are each independently N or CR^{25};

Y^5 is S, O, or NH; and

each R^{25} is independently selected from H, optionally substituted $C_{(1-6)}$-alkyl, optionally substituted fluoroalkyl, and halogen.

In some embodiments, each R^{25} is H.

In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by one of the following structures:


, and

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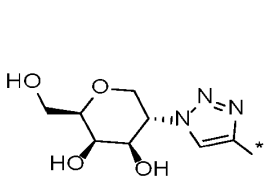
In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by one of the following structures: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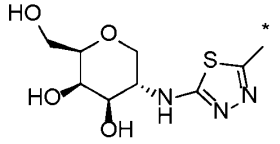
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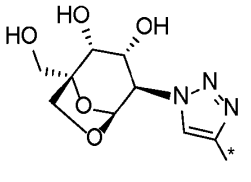
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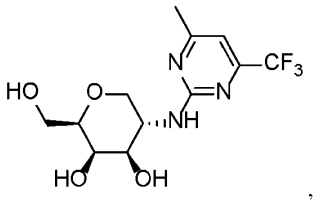
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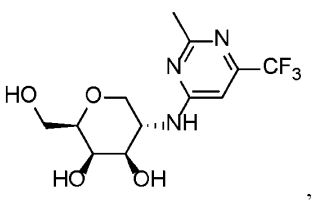
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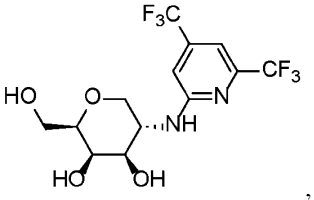
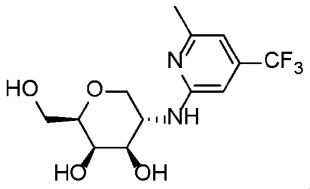

, and

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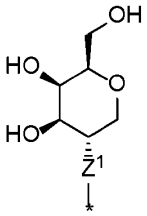
In some embodiments of Formula IIb, R^1 R^3, R^4, and R^{11} are H, and R^6 is OH:

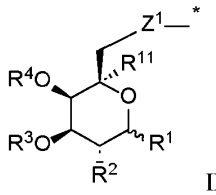


wherein Z^1 is -NH-, -CH_2-, -S- or -O-.

6-linked ASGPR ligand moieties

In some embodiments, the ASGPR binding moiety (X) is linked via the 6-postion of the sugar analog. In some embodiments, the ASGPR binding moiety (X) has a reduced ring carbon at the 1-position relative to a galactosamine derived sugar.

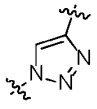
In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by Formula IIc:



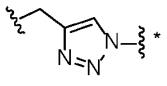
IIc

wherein R^1-R^4 and Z^1 are as defined herein.

In some embodiments of Formula IIc, Z^1 is selected from -O-, -S-, -CONR^{21}-, and optionally substituted -(C(R^{22})$_2$)$_q$- heteroarylene, wherein q is 0 or 1. In some embodiments, Z^1 is -O-. In certain other cases, Z^1 is optionally substituted -(C(R^{22})$_2$)$_q$-triazole wherein q is 0 or 1.

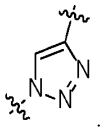
In some embodiments, Z^1 is .

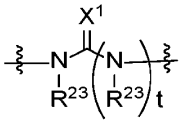
In some embodiments of Formula IIc, Z^1 is -Z^{11}-A^1-, wherein -A^1- is or optionally substituted -A^1- or optionally substituted arylene. In some embodiments, -A^1- is an optionally substituted heteroarylene. In some embodiments, the heteroarylene is a 5 or 6-membered heteroarylene. In some embodiments, the heteroarylene is a 5-membered heteroarylene. In some embodiments, the 5-membered heteroarylene is a triazole. In some embodiments, the triazole is a 1,2,3-triazole moiety. In some embodiments, Z^{11} is -C(R^{22})$_2$. In some embodiments, at least one R^{22} is H. In some embodiments, both R^{22} are H. In some embodiments Z^{11} is -O-. In some embodiments, Z^{11} is -S-. In certain other cases, Z^{11} is -N(R^{21}), where R^{21} is H or C$_{1-3}$ alkyl. In some embodiments, Z^1 is -C(R^{22})$_2$-triazole-.

In some embodiments, Z^1 is .

In some embodiments of Formula IIc, Z^1 is Z^{11}. In some embodiments, Z^{11} is -C(R^{22})$_2$. In some embodiments, at least one R^{22} is H. In some embodiments, both R^{22} are H, and Z^{11} is -CH$_2$-. In some

embodiments Z^{11} is -O-. In some embodiments, Z^{11} is -S-. In certain other cases, Z^{11} is -N(R^{21}), where R^{21} is H or (C_1-C_3)alkyl.

In some embodiments of Formula IIc, Z^1 is monocyclic 5 or 6-membered heteroarylene or arylene. In some embodiments, Z^1 is .

In some embodiments of Formula IIc, Z^1 is selected from -O-, -S-, -C(R^{22})$_2$-, -N(R^{21})-, -CON(R^{21})-, and ;

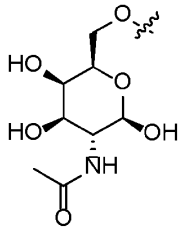
wherein:

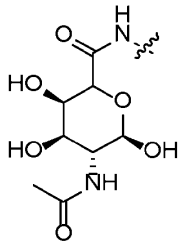
X^1 is O or S;

t is 0 or 1;

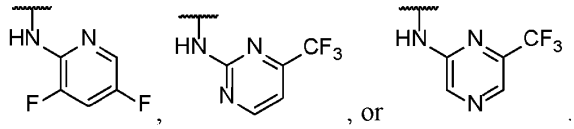
R^{21} and each R^{23} is independently selected from H, and optionally substituted C_1-$_6$alkyl (e.g., C_{1-3}-alkyl, such as methyl); and

each R^{22} is independently selected from H, halogen (e.g., F) and optionally substituted C_1-$_6$alkyl.

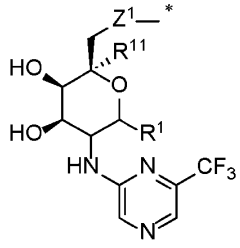
In some embodiments, the compound of Formula IIc is .

In some embodiments, the compound of Formula IIc is .

In some embodiments, R^2 is:



In some embodiments, the ASGPR ligand moiety (X) of the compounds of this disclosure can be described by Formula IIc-1:



IIc-1

wherein:

R^1 is -H, -OH, optionally substituted (C_1-C_6)alkyl, or -OCH_3, and R^{11} is hydrogen; or R^1 and R^{11} together with the carbon atoms to which each is attached form a bridging moiety;

Z^1 is a linking moiety selected from -Z^{11}-, -Z^{11}-A^1-*, -A^2-, -$NR^{21}CO$-*, - $CONR^{21}$-*, -$NR^{21}SO_2$*-, -SO_2NR^{21}-*, -$NR^{21}C(=O)NR^{21}$-, and -$NR^{21}C(=S)NR^{21}$-; where " * " represents a point of connection of Z^1 to the linker (L);

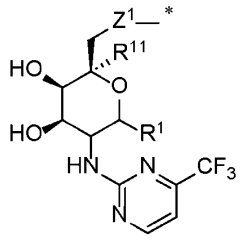
-Z^{11}- is -O-, -S-, -$N(R^{21})$-, or -$C(R^{22})_2$;

-A^1- and -A^2- are optionally substituted heterocyclylene, optionally substituted arylene, or optionally substituted heteroarylene;

each R^{21} is independently selected from H, optionally substituted (C_1-C_6)alkyl, -COR, and optionally substituted heteroaryl; and

each R^{22} is independently selected from H, halogen, and optionally substituted (C_1-C_6)alkyl.

In some embodiments, the ASGPR ligand moiety (X) of the compounds of this disclosure can be described by Formula IIc-2:



IIc-2

wherein:

R^1 is -H, -OH, optionally substituted (C_1-C_6)alkyl, or -OCH_3, and R^{11} is hydrogen; or R^1 and R^{11} together with the carbon atoms to which each is attached form a bridging moiety;

Z^1 is a linking moiety selected from -Z^{11}-, -Z^{11}-A^1-*, -A^2-, -$NR^{21}CO$-*, - $CONR^{21}$-*, -$NR^{21}SO_2$*-, -SO_2NR^{21}-*, -$NR^{21}C(=O)NR^{21}$-, and -$NR^{21}C(=S)NR^{21}$-; where " * " represents a point of connection of Z^1 to the linker (L);

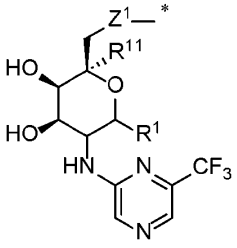
-Z^{11}- is -O-, -S-, -$N(R^{21})$-, or -$C(R^{22})_2$;

-A^1- and -A^2- are optionally substituted heterocyclylene, optionally substituted arylene, or optionally substituted heteroarylene;

each R^{21} is independently selected from H, optionally substituted (C_1-C_6)alkyl, -COR, and optionally substituted heteroaryl; and

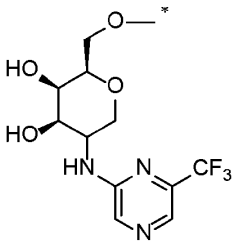
each R^{22} is independently selected from H, halogen, and optionally substituted (C_1-C_6)alkyl.

In some embodiments, the ASGPR ligand moiety (X) of the compounds of this disclosure can be described by Formula IIc-1A:



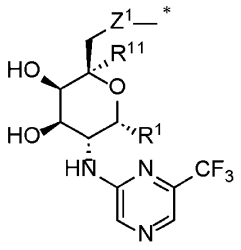
IIc-1A.

In some embodiments, the ASGPR ligand moiety (X) of the compounds of this disclosure can be described by Formula IIc-2A:

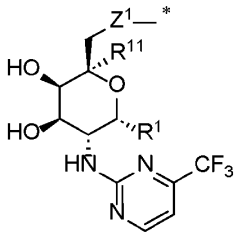


IIc-2A.

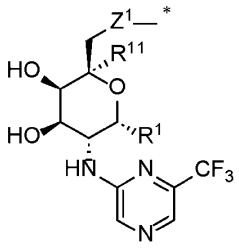
In some embodiments, the ASGPR ligand moiety (X) of the compounds of this disclosure can be described by Formula a-IIc-1, a-IIc-2, a-IIc-1A, or a-IIc-2A:



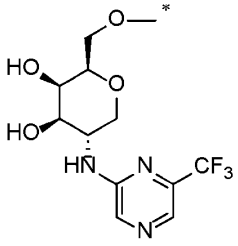
a-IIc-1



a-IIc-2

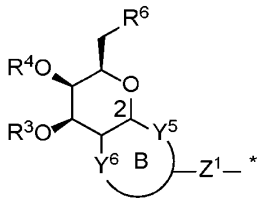


a-IIc-1A



a-IIc-2A.

In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by Formula IId:



IId

wherein:

R^6, R^4, R^3, and Z^1 are as defined herein;

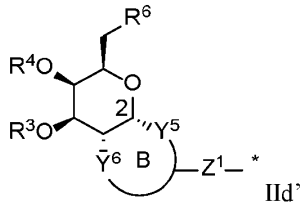
Y^6 and Y^5 are each independently selected from -O-, -S-, NR^{21}-, and $-C(R^{22})_2$;

R^{21} is selected from H, optionally substituted C_{1-6}alkyl, and $-C(O)R^{22}$;

each R^{22} is independently selected from H, halogen and optionally substituted C_{1-6}alkyl; and

ring B is a 5 or 6-membered optionally substituted cyclic group. In some embodiments of Formula IId, Y^5 is connected to the sugar ring via an alpha configuration. In some embodiments of Formula IId, Y^5 is connected to the sugar ring via a beta configuration.

In some embodiments, the ASGPR binding moiety (X) of the compounds of this disclosure can be described by Formula IId':



IId'

wherein:

R^6, R^4, R^3, and Z^1 are as defined herein;

Y^5 and Y^6 are each independently selected from -O-, -S-, NR^{21}-, and $-C(R^{22})_2$;

R^{21} is selected from H, optionally substituted $(C_1$-$C_6)$alkyl, and $-C(O)R^{22}$;

each R^{22} is independently selected from H, halogen and optionally substituted $(C_1$-$C_6)$alkyl; and

ring B is a 5 or 6-membered optionally substituted cyclic group.

In some embodiments of Formula IId-IId', Y^5 is O. In some embodiments, Y^5 is S. In some embodiments, Y^5 is $-NR^{21}$-. In some embodiments, Y^5 is $-C(R^{22})_2$ and each R^{22} is H.

In some embodiments of Formula IId-IId', Y^6 is $-NR^{21}$- where R^{21} is H. In some embodiments, Y^6 is $-NR^{21}$- where R^{21} is $-C(O)R^{22}$. In some cases, R^{22} is methyl.

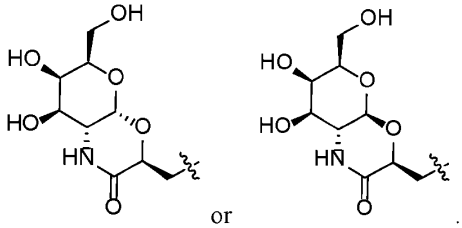
In some embodiments of Formula IId-IId', the B ring is a 5 or 6-membered heterocycle. In some cases, the B ring is a 5-membered heterocycle. In some cases, the B ring is a 6-membered heterocycle.

In some embodiments of Formula IId-IId', Z^1 is Z^{11}, where Z^{11} is selected from -O-, -S-, NR^{21}-, and $-C(R^{22})_2$. In some cases, Z^1 is -O-. In some cases, Z^1 is -S-. In some cases, Z^1 is NR^{21} where R^{21} is H. In some cases, Z^1 is $-C(R^{22})_2$ where each R^{22} is H.

In some embodiments of Formula IId-IId', Z^1 is optionally substituted Z^{11}-heteroarylene or optionally substituted Z^{11}-arylene. In some embodiments, Z^1 is CH_2-heteroarylene or CH_2-arylene. In some embodiments of Formula IId-IId', Z^1 is optionally substituted amide. In some embodiments of

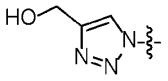
Formula IId-IId', Z^1 is optionally substituted sulfonamide. In some embodiments of Formula IId-IId', Z^1 is optionally substituted urea or optionally substituted thiourea.

In some embodiments, X has one of the following structures:

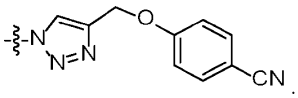


or .

In some embodiments of Formula IIa, IIb, or IId, R^6 is OH. In certain other cases, R^6 is -OC(O)R. In some embodiments, R^6 is -C(O)NHR, where R is an optionally substituted alkyl. In some embodiments, R terminates in an alkenyl or an alkynyl group. In certain other cases R^6 is optionally substituted triazole.

In some embodiments, the triazole is .

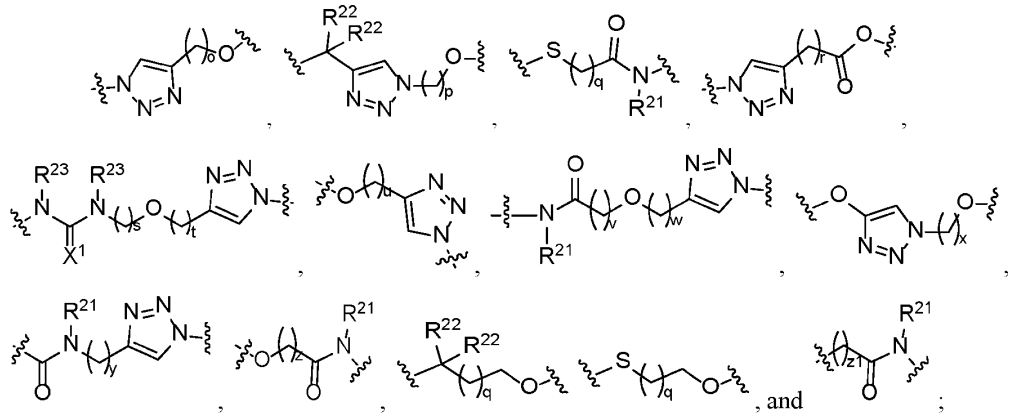
In some embodiments of Formula IIa or IIc, R^2 is $-NHCOCH_3$. In some embodiments, R^2 is $-NHCOCF_3$. In certain other embodiments, R^2 is $-NHCOCH_2CF_3$. In some embodiments, R^2 is -OH. In some embodiments, R^2 is an optionally substituted triazole.

In some embodiments, the triazole is .

In some embodiments, when R^6 or R^2 is a substituted triazole, the triazole is a 1,2,3-trizole, and the substituent is at the 4 or 5-position. In some embodiments, the substituent on the triazole moiety includes but is not limited to, an optionally substituted C_{1-6}alkyl, optionally substituted C_{1-6}alkoxy, optionally substituted aryl, optionally substituted heteroaryl, optionally substituted alkaryl, and an optionally substituted alkyheteroaryl. It will be understood that any convenient substituent can be included in the triazole moiety, see, e.g., triazole moieties disclosed in Mamidayala et al, *J. Am. Chem. Soc.* 2012, 134, 1978-1981.

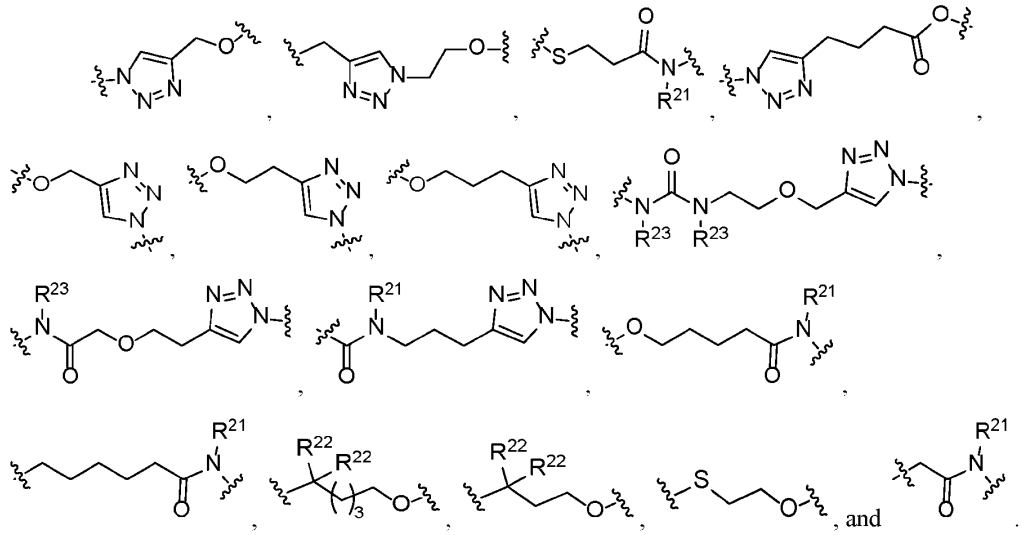
It is understood that the Z^1, Z^{11}, and Z^{11}-Ar linking moieties can be considered part of the X group of Formula I. In the ASGPR binding moieties (X) as described herein, $-Z^1-$ can be linked to an $-L^1-$ moiety (e.g., of the linker as described herein) via a variety of bonds and linking moieties, depending on the method of preparation.

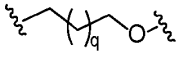
In some embodiments, the subject compounds comprise a -Z^1-L^1- moiety selected from:

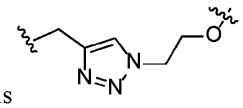


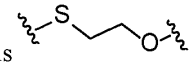
wherein each R^{21} is independently selected from H, and optionally substituted (C_1-C_6)alkyl; each R^{22} is independently selected from H, halogen (e.g., F) and optionally substituted (C_1-C_6)alkyl; and o, p, q, r, s, t, u, v, w, x, y, z and z1 are each independently 1 to 6. In some embodiments, o, p, q, r, s, t, u, v, w, x, y, z and z1 are each independently 1 to 6.

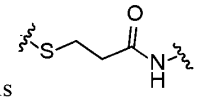
In some embodiments, the subject compounds comprise a -Z^1-L- group selected from:



In some embodiments, the Z^1-L^1- group is , where q is 1-3. In some embodiments, q is 1. In some embodiments, q is 2. In some embodiments, q is 3.

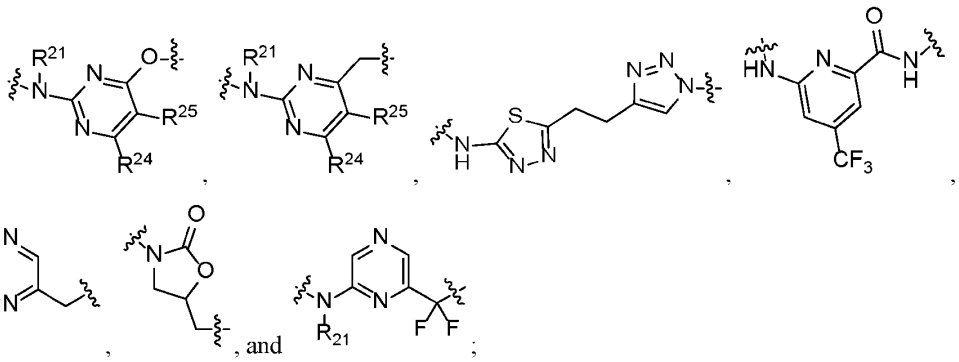
In some embodiments, the Z^1-L^1- group is .

In some embodiments, the Z^1-L^1- group is .

In some embodiments, the Z^1-L^1- group is .

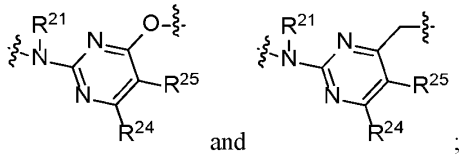
In some embodiments, -Z^1-L^1- comprises an optionally substituted -NH-heteroarylene-. In some embodiments the heteroarylene is a triazole. In some embodiments, the heteroarylene is pyridine. In some embodiments, the heteroarylene is pyrimidine. In some embodiments, the heteroarylene is thiadiazole.

In some embodiments, the -Z^1-L^1- comprises a group selected from:



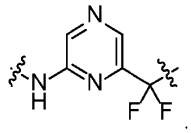
wherein each R^{21} is independently selected from H, optionally substituted (C_1-C_6)alkyl, and optionally substituted acyl; and R^{24} and R^{25} are each independently selected from H, optionally substituted $C_{(1-6)}$-alkyl, optionally substituted fluoroalkyl, and halogen.

In some embodiments, the -Z^1-L^1- comprises a group selected from:

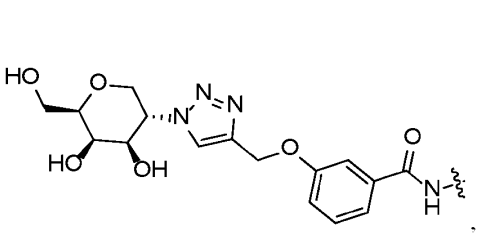
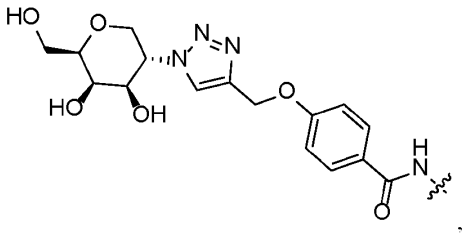
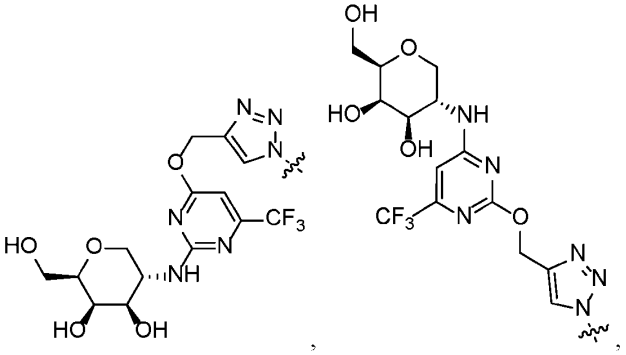
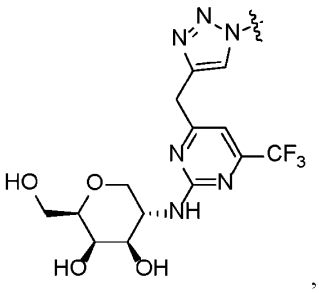
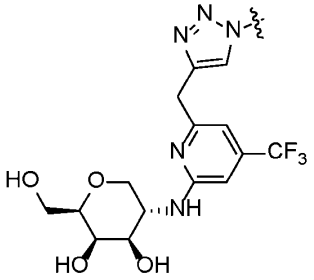
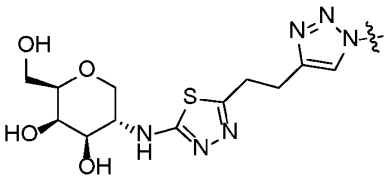
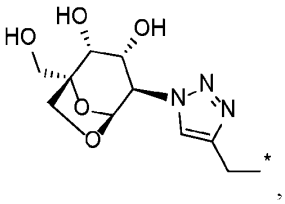


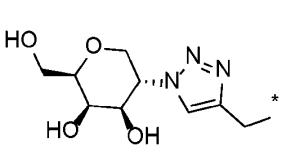
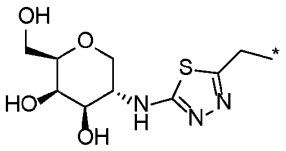
wherein R^{24} and R^{25} are each independently selected from H, optionally substituted $C_{(1-6)}$-alkyl, optionally substituted fluoroalkyl, and halogen; and each R^{21} is independently selected from H, optionally

substituted (C_1-C_6)alkyl, and optionally substituted acyl. In some embodiments, R^{21} is H. In some embodiments, R^{24} is $C_{(1-3)}$-alkyl, or $C_{(1-3)}$-fluoroalkyl. In some cases, the fluoroalkyl is CF_3. In some embodiments, R^{25} is $C_{(1-3)}$-alkyl, or $C_{(1-3)}$-fluoroalkyl. In some cases, the fluoroalkyl is CF_3.

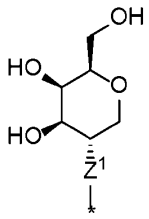
In some embodiments, -Z^1-L^1- is .

It is understood that a variety of substituents and chemistries can be utilized to connect a particular X ligand moiety (e.g., as described herein) to an adjacent linker. In some embodiments, a linking moiety of the linker comprises a triazole that derives from a Click chemistry conjugation. In some embodiments, the ASGPR ligand moiety (X) is attached to a linking moiety as shown in one of the following structures:

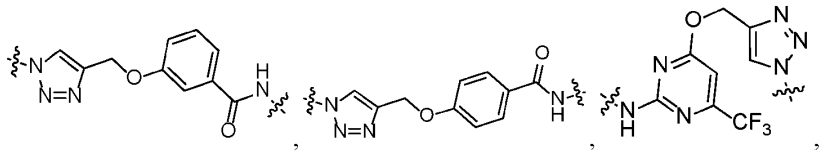
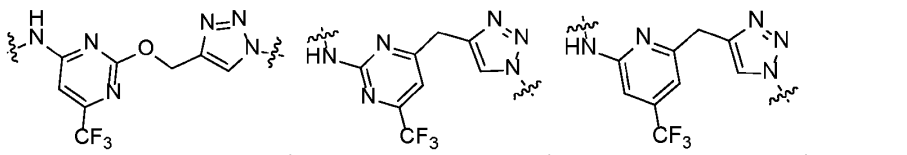
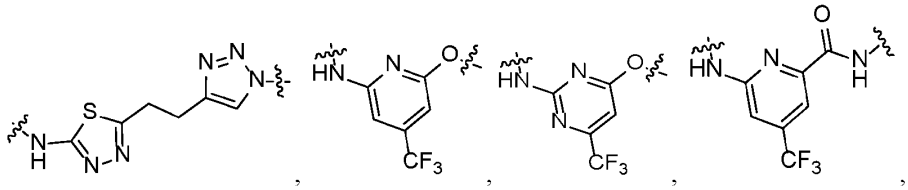
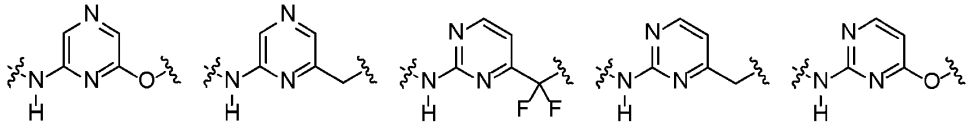
,
,
,
,
,
,
.

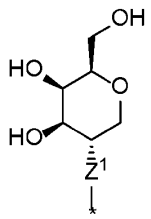
In some embodiments, R^1 R^3, R^4, and R^{11} are H, and R^6 is OH:



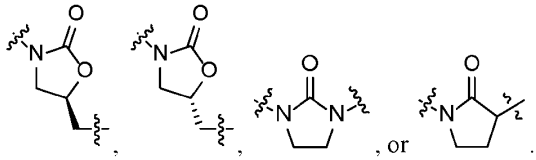
wherein Z^1 is triazole, -NH-heteroaryl (e.g., -NH- attached to pyridine or pyrimidine), -NH-, -O-, or $-CH_2-$, and/or Z^1 is attached to a linking moiety as shown in one of the following structures:


In some embodiments, of Formula Iib, R^1 R^3, R^4, and R^{11} are H, and R^6 is OH:



wherein Z^1 is attached to a linking moiety as shown in one of the following structures:



1.1.2 Prodrugs

Aspects of this disclosure include prodrugs of any of the ASGPR ligand moieties described herein that are incorporated into the linker compounds and conjugates of this disclosure. The term "prodrug" refers to an agent which is converted into the drug in vivo by some physiological or chemical process (e.g., a prodrug on being brought to the physiological pH is converted to the desired drug form). Prodrugs forms of any of the ASGPR ligand moieties described herein can be useful because, for example, can lead to particular therapeutic benefits as a consequence of an extension of the half-life of the resulting compound or conjugate in the body or a reduction in the active dose required. Prodrugs can also be useful in some situations, as they may be easier to administer than the parent drug. They may, for instance, be bioavailable by oral administration whereas the parent drug is not. The prodrug may also have improved solubility in pharmacological compositions over the parent drug.

A prodrug derivative of a ASGPR ligand moiety generally includes a labile promoiety substituent at a suitable site of the moiety. The promoiety refers to the group that is removed by enzymatic or chemical reactions, when a prodrug is converted to the drug *in vivo*.

In some embodiments, the promoiety is a group, such as an optionally substituted alkanoyl, attached via an ester linkage to a hydroxyl group of the moiety. In some embodiments, a prodrug derivative of one or more of the hydroxyl groups of the sugar ring of the ASGPR ligand moiety may be incorporated into the compounds. For example, an ester promoiety can be incorporated at one or more of the hydroxyl groups at the 3 and/or 4 positions of the core sugar ring (e.g., as described in Formula (II)).

In some embodiments, the hydroxyl groups at the 3 and 4 positions of the core sugar ring are cyclically linked to form a promoiety (e.g., as described herein).

In some embodiments, the promoiety is the part of an ester group attached to a hydroxyl of X, such as $-C(O)CH_3$, $-C(O)CH(CH_3)_2$, $-C(O)C(CH_3)_3$, or $-CH_2OC(O)C(CH_3)_3$. In some embodiments, the promoicty is $-CH_2OC(O)C(CH_3)_3$. In some embodiments, a promoiety cyclically links two adjacent hydroxyl groups via a carbonate linkage, *i.e.,* a cyclic carbonate.

1.1.3 Linker

The terms "linker," "linking moiety," and "linking group" are used interchangeably and refer to a linking moiety that covalently connects two or more moieties or compounds, such as ligands and other moieties of interest. In some cases, the linker is divalent and connects two moieties. In some embodiments, the linker is a branched linking group that is trivalent or of a higher multivalency. In some cases, the linker that connects the two or more moieties has a linear or branched backbone of 500 atoms or less (such as 400 atoms or less, 300 atoms or less, 200 atoms or less, 100 atoms or less, 80 atoms or less, 60 atoms or less, 50 atoms or less, 40 atoms or less, 30 atoms or less, or even 20 atoms or less) in length, e.g., as measured between the two or more moieties. A linking moiety may be a covalent bond that connects two groups or a linear or branched chain of between 1 and 500 atoms in length, for example of about 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, 100, 150, 200, 300, 400 or 500 atoms in length, where the linker may be linear, branched, cyclic or a single atom. In some embodiments, one, two, three, four, five or more, ten or more, or even more carbon atoms of a linker backbone may be optionally substituted with heteroatoms, e.g., sulfur, nitrogen or oxygen heteroatom. In certain instances, when the linker includes a PEG group, every third atom of that segment of the linker backbone is substituted with an oxygen. The bonds between backbone atoms may be saturated or unsaturated, usually not more than one, two, or three unsaturated bonds will be present in a linker backbone. The linker may include one or more substituent groups, for example an alkyl, aryl or alkenyl group. A linker may include, without limitations, one or more of the following: oligo(ethylene glycol), ether, thioether, disulfide, amide, carbonate, carbamate, tertiary amine, alkyl which may be straight or branched, e.g., methyl, ethyl, n-propyl, 1-methylethyl (iso-propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), and the like. The linker backbone may include a cyclic group, for example, an aryl, a heterocycle, a cycloalkyl group or a heterocycle group, where 2 or more atoms, e.g., 2, 3, or 4 atoms, of the cyclic group are included in the backbone.

In some embodiments, a "linker" or "linking moiety" is derived from a molecule with two reactive termini, one for conjugation to a moiety of interest (Y), e.g., a biomolecule (e.g., an antibody) and the other for conjugation to a moiety (noted as X) that binds to a ASGPR cell surface receptor. When

Y is a polypeptide, the polypeptide conjugation reactive terminus of the linker is in some cases a site that is capable of conjugation to the polypeptide through a cysteine thiol or lysine amine group on the polypeptide, and so is can be a thiol-reactive group such as a maleimide or a dibromomaleimide, or as defined herein, or an amine-reactive group such as an active ester (e.g., perfluorophenyl ester or tetrafluorophenyl ester), or as defined herein.

In some embodiments of the formula described herein, the linker (i.e., L^1-L^6) comprises one or more straight or branched-chain carbon moieties and/or polyether (e.g., ethylene glycol) moieties (e.g., repeating units of -CH_2CH_2O-), and combinations thereof. In some embodiments, these linkers optionally have amide linkages, urea or thiourea linkages, carbamate linkages, ester linkages, amino linkages, ether linkages, thioether linkages, sulfhydryl linkages, heteroaryl linkages, or other hetero functional linkages. In some embodiments, the linker comprises one or more of carbon atoms, nitrogen atoms, sulfur atoms, oxygen atoms, and combinations thereof. In some embodiments, the linker comprises one or more of an ether bond, thioether bond, amine bond, amide bond, carbon-carbon bond, carbon-nitrogen bond, carbon-oxygen bond, carbon-sulfur bond, and combinations thereof. In some embodiments, the linker comprises a linear structure. In some embodiments, the linker comprises a branched structure. In some embodiments, the linker comprises a cyclic structure. In some embodiments, the linker comprises one or more heteroaryl cyclic structures, e.g., a triazole, such as a 1,2,3-traizole.

In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 4 to 500 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 4 to 50 consecutive atoms. In some embodiments, linker separates X and the Y-B complex by a chain of 6 to 50 consecutive atoms, by a chain of 11 to 50 consecutive atoms, by a chain of 16 to 50 consecutive atoms, by a chain of 21 to 50 consecutive atoms, by a chain of 26 to 50 consecutive atoms, by a chain of 31 to 50 consecutive atoms, by a chain of 36 to 50 consecutive atoms, by a chain of 41 to 50 consecutive atoms, or by a chain of 46 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 6 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 11 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 16 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 21 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 26 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 31 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 36 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 41 to 50 consecutive

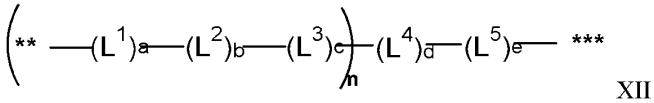
atoms. In some embodiments, the linker (i.e., L^1-L^6) separates X and the Y-B complex by a chain of 46 to 50 consecutive atoms.

In some embodiments, the linker (i.e., L^1-L^6) is a chain of 5 to 500 consecutive atoms separating X and the Y-B complex and which comprises an optionally substituted arylene linked to X, optionally substituted heteroarylene linked to X, optionally substituted alkylene linked to X, or heteroatom linked to X. In some embodiments, the linker (i.e., L^1-L^6) is a chain of 7 to 500 consecutive atoms separating X and the Y-B complex and which comprises an optionally substituted arylene linked to X, optionally substituted heteroarylene linked to X, optionally substituted alkylene linked to X, or heteroatom linked to X.

In some embodiments, the linker (i.e., L^1-L^6) is a chain of 10 to 500 consecutive atoms separating X and the Y-B complex and which comprises an optionally substituted arylene linked to X, optionally substituted heteroarylene linked to X, optionally substituted alkylene linked to X, or heteroatom linked to X. In some embodiments, the linker (i.e., L^1-L^6) is a chain of 15 to 400 consecutive atoms separating X and Y and which comprises an optionally substituted arylene linked to X, optionally substituted heteroarylene linked to X, optionally substituted alkylene linked to X, or heteroatom linked to X.

In some embodiments, the linker (i.e., L^1-L^6) is a chain of 16 to 400 consecutive atoms separating X and Y (or Z) and which comprises an optionally substituted arylene linked to X, optionally substituted heteroarylene linked to X, optionally substituted alkylene linked to X, or a heteroatom linked to X.

In some embodiments, L is of Formula XII:



XII

wherein

each L^1 to L^5 is independently a linking moiety which together provide a linear or branched linker between Z^1 and Y;

a, b, c, d, and e are each independently 0, 1, or 2;

** represents the point of attachment of L^1 to X via Z^1; and

*** represents the point of attachment to the Y-B complex;

wherein:

when n is 1, a is 1, and c is 0; and

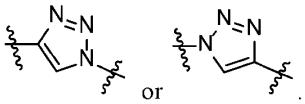
when n is >1, a is 1, and c is 1.

It is understood that the linker may be considered as connecting directly to a Z^1 group of a ASGPR binding moiety (X) (e.g., as described herein). In some embodiments of any of Formula Ia-Ip, the linker may be considered as connecting directly to the Z^1 group. Alternatively, the $-Z^1-L^1-$ group (e.g., as described herein) can be considered part of a linking moiety that connects L to Y. The disclosure is meant to include all such configurations of ASGPR binding moiety (X) and linker (L).

In some embodiments of Formula XII, the linking moiety L^1 includes a linear backbone of 6 to 40 consecutive atoms, such as 10 to 40, 10 to 30, 16 to 30, or 20 to 30 consecutive atoms. In some embodiments of Formula XII, the linking moiety L^1 includes a linear backbone of each L^1 comprises a linear backbone of 6 to 20 consecutive atoms, such as 6 to 16 consecutive atoms, such as 8, 9, 10, 11, 12, 13, 14, 15 or 16 consecutive atoms.

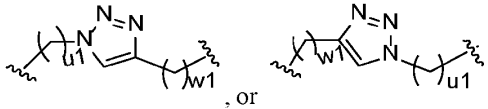
In some embodiments, the linking moiety of Formula XII includes one or repeating ethylene glycol moieties (e.g., $-CH_2CH_2O-$ or $-OCH_2CH_2-$). In some embodiments, the linking moiety of Formula XII includes 1 to 10 ethylene glycol moieties, such as 1, 2, 3, 4, 5 or 6 ethylene glycol moieties.

In some embodiments, the linking moiety of Formula XII includes one or more triazole (e.g., 1,2,3-triazole) containing linking moieties. It is understood that the triazole may be derived from an azido-alkyne click chemistry and thus have two possible orientations depending on the method of synthesis:



or .

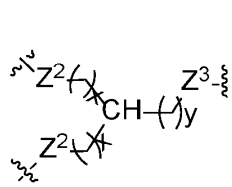
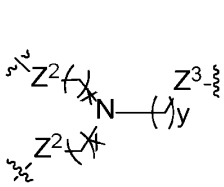
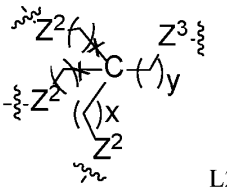
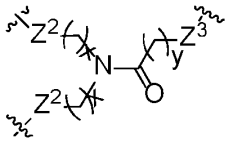
In some embodiments, the triazole containing linking moiety is:



, or

wherein w1 and u1 are independently 0 to 12, such as 0, 1, 2, 3, 4, 5 or 6.

In some embodiments of the linker of Formula XII, d is 1 and the linking moiety L^4 is selected from one of Formula L2A-L2D:


L2A,

L2B,

L2C, and

L2D,

wherein:

each Z^2 and Z^3 is independently absent or selected from -NHCO-, -CONH-, -CO-, -O-, -NH-, and -NCH_3-;

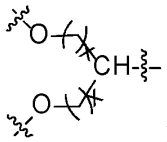
x is 1 to 12 (e.g., 1 to 6, or 1 to 3); and

y is 0 to 12 (e.g., 1 to 6, or 1 to 3).

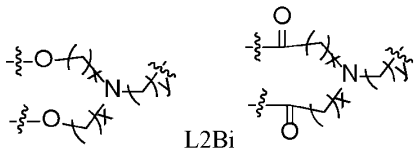
In some embodiments of any one of Formula L2A-L2D, Z^2 is -NHCO-. In some embodiments of any one of Formula L2A-L2D, Z^2 is -CONH-. In some embodiments of any one of Formula L2A-L2D, Z^2 is -CO-. In some embodiments of any one of Formula L2A-L2D, Z^2 is -O-. In some embodiments of any one of Formula L2A-L2D, Z^2 is -NH-. In some embodiments of any one of Formula L2A-L2D, Z^2 is -NCH_3-. In some embodiments of any one of Formula L2A-L2D, Z^2 is absent.

In some embodiments of any one of Formula L2A-L2D, Z^3 is -NHCO-. In some embodiments of any one of Formula L2A-L2D, Z^3 is -CONH-. In some embodiments of any one of Formula L2A-L2D, Z^3 is -CO-. In some embodiments of any one of Formula L2A-L2D, Z^3 is -O-. In some embodiments of any one of Formula L2A-L2D, Z^3 is -NH-. In some embodiments of any one of Formula L2A-L2D, Z^3 is - NCH_3-. In some embodiments of any one of Formula L2A-L2D, Z^3 is absent.

In some embodiments of Formula L2A, Z^2 is -O-, y is 0, and the linking moiety is of Formula L2Ai:

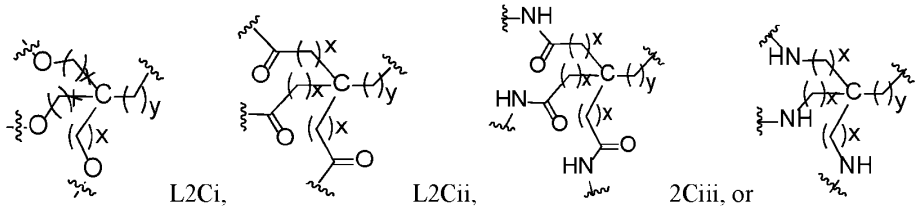

L2Ai.

In some embodiments of Formula L2B, Z^2 is -O- or -CO-, and the linking moiety is of Formula L2Bi or L2Bii:



L2Bi L2Bii.

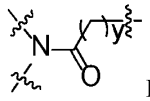
In some embodiments of Formula L2C, Z^2 is -O-, -C(O)-, -NHC(O)-, or -NH-, and the linking moiety is of Formula L2Ci, L2Cii, L2Ciii, or L2Civ:



L2Ci, L2Cii, 2Ciii, or L2Civ.

In some embodiments, of any one of Formula L2A- L2Civ, x is 1 to 6. In some embodiments, x is 1 to 3. In some embodiments, x is 1. In some cases, x is 2. In some embodiments, x is 3.

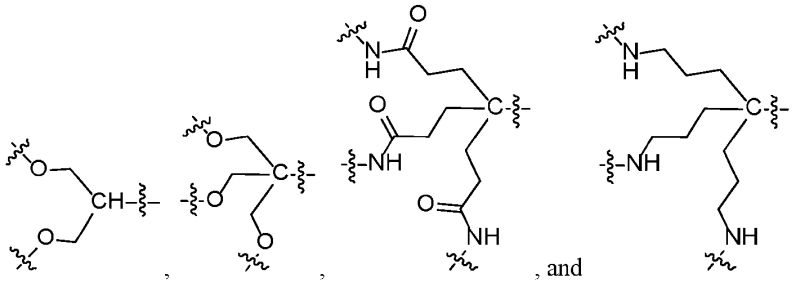
In some embodiments of Formula L2D, Z^2 is absent and the linking moiety is Formula L2Di:



L2Di.

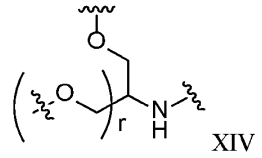
In some embodiments of any one of Formula L2A-L2Di, y is 0 to 6. In some embodiments, y is 0 to 3. In some embodiments, y is 0. In some embodiments, y is 1. In some embodiments, y is 2. In some embodiments, y is 3.

In some embodiments of Formula XII, d is 1 and the linking moiety L^4 is selected from:



, , , and .

In some embodiments of Formula XII, d is 1 and the linking moiety L^4 is Formula XIV:



XIV

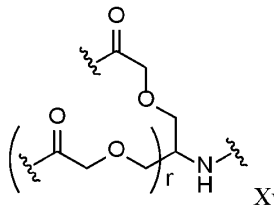
wherein:

r is 1 or 2; and

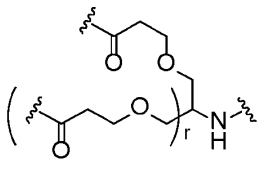
when n is 2, r is 1,

when n is 3, r is 2.

In some embodiments of the linker of formula (XI), b is 1 and the linking moiety L^2 is Formula Xva or XVb:



Xva,



XVb

wherein:

r is 1 or 2; and

when n is 2, r is 1,

when n is 3, r is 2.

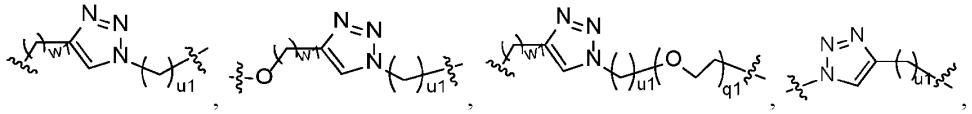
In some embodiments L^2 is of Formula XIIIa or XIIIb and L^2 includes two 2 or more amino acid residues (e.g., 3 or more, or 4 or more amino acid residues, linear or dendrimer). In some embodiments, L^2 includes 4 or more amino acid residues that are branched linking moieties selected from Lys, Orn, Asp, Glu, Ser, and Cys (e.g., where the sidechain, amino and carboxylic acid are each linked to an adjacent moiety).

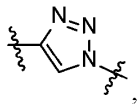
In some embodiments, the linking moiety of Formula XVI includes a linear backbone of 6 to 40 consecutive atoms, such as 10 to 40, 10 to 30, or 20 to 30 consecutive atoms.

In some embodiments, the linking moiety of Formula XVI includes repeating ethylene glycol moieties (e.g., $-CH_2CH_2O-$ or $-OCH_2CH_2-$). In some embodiments, the linking moiety of Formula XVI includes 2 to 20 ethylene glycol moieties, such as 2 to 15, 2 to 10, 3 to 20, 3 to 15, 3 to 10, 4 to 15, 5 to 15 or 5 to 10 ethylene glycol moieties. In some instances, the linking moiety of Formula XVI includes 2 or

more ethylene glycol moieties, such as 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, or even more ethylene glycol moieties.

In some embodiments, the linking moiety of Formula XVI includes one or more triazole linking moieties. In some instances, the linker includes one or more 1,2,3-triazole linking moieties. In some embodiments, the one or more 1,2,3-triazoel moieties is selected from one of the following structures:

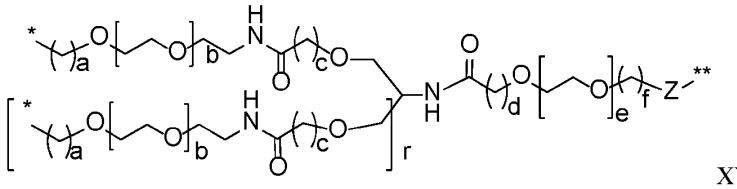




and , wherein w1, u1 and q1 are independently 1 to 25 (e.g., 1 to 12, such as 1 to 6).

In some embodiments, the linking moiety L^3 includes C_{10}-C_{20}-alkylene (e.g., C_{12}-alkylene), or $-(OCH_2CH_2)_p-$, where p is 1 to 25, such as 3 to 25, 5 to 24, 7 to 25, 10 to 25, 15 to 25 or 20 to 24.

In some embodiments, the linker (i.e., L^1-L^6) comprises Formula XVII:



XVII

wherein:

a is 0 to 12 (e.g., 2 to 6, or 2, or 3);

b is 1 to 6 (e.g., 1, 2, or 3);

c is 1 to 6 (e.g., 1, 2, or 3);

r is 1 or 2;

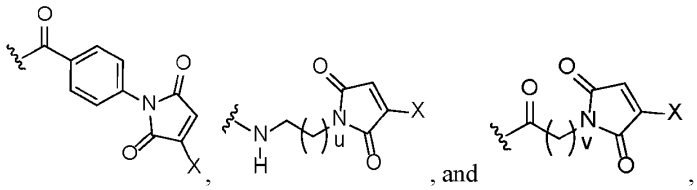
d is 1 to 6 (e.g., 1, 2, or 3);

e is b is 1 to 6 (e.g., 1, 2, or 3);

f is 1 to 6 (e.g., 1, 2, or 3); and

Z is a residual moiety resulting from the covalent linkage of a chemoselective ligation group (e.g., as described herein) of a linker precursor to a compatible group of Y-B.

In some embodiments of the Formula XVII, Z or the terminal L^6 is a residual moiety resulting from the covalent linkage (e.g., via a thioether bond) of a thiol-reactive chemoselective ligation group to one or more cysteine residue(s) of Y-B. In some embodiments, the thiol-reactive chemoselective ligation group includes maleimide, bromomaleimide, haloacetamide, vinyl sulfone, or thiolactone. In some embodiments, the thiol-reactive group is selected from one of the following structures:



wherein:

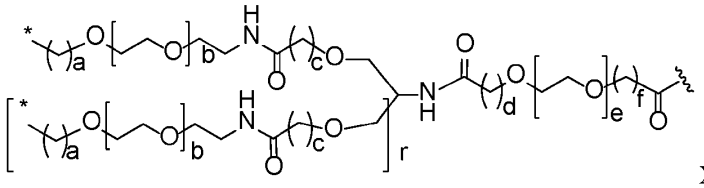
u is 1 to 11 (e.g., 1 to 5);

v is 1 to 11 (e.g., 1 to 5); and

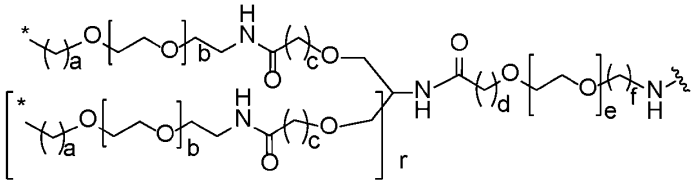
X is H or Br.

In some embodiments of formula XVII, Z is a residual moiety resulting from the covalent linkage (e.g., via an amide bond) of an amine-reactive chemoselective ligation group to one or more lysine residue(s) of Y-B. In some embodiments, the amine-reactive chemoselective ligation group includes an active ester (e.g., N-hydroxysuccinimidyl (NHS) ester, sulfo-NHS ester, pentafluorophenyl (PFP) ester, tetrafluorophenyl (TFP) ester, or the like).

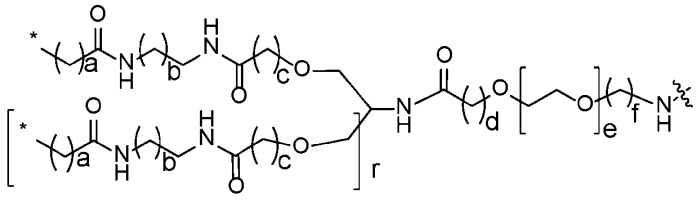
In some embodiments, the linker (i.e., L^1-L^6) comprises one of Formula XVIIIa-XVIIIc:



XVIIIa,



XVIIIb, or



XVIIIc,

wherein:

a is 0 to 12 (e.g., 2 to 6, or 2, or 3);

b is 1 to 6 (e.g., 1, 2, or 3);

c is 1 to 6 (e.g., 1, 2, or 3);

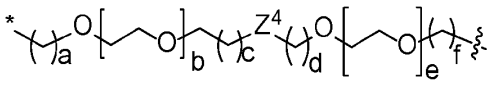
r is 1 or 2;

d is 1 to 6 (e.g., 1, 2, or 3);

e is b is 1 to 6 (e.g., 1, 2, or 3); and

f is 1 to 6 (e.g., 1, 2, or 3).

In some embodiments, the linker (i.e., L^1-L^6) comprises L^A:



(L^A)

wherein:

Z^4 is selected from -NHC(O)NH-, -NHC(O)-, -C(O)NH-, -O-, -NH-;

a is 0 to 12 (e.g., 2 to 6, or 2, or 3);

b is 1 to 6 (e.g., 1, 2, or 3);

c is 1 to 6 (e.g., 1, 2, or 3);

d is 1 to 6 (e.g., 1, 2, or 3);

e is b is 1 to 6 (e.g., 1, 2, or 3); and

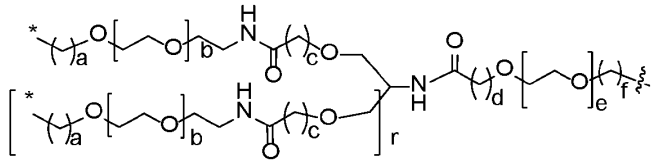
f is 1 to 6 (e.g., 1, 2, or 3).

In some embodiments of L^A, Z^4 is -NHC(O)NH-. In some cases, Z^4 is -NHC(O)-. In some cases, Z^4 is -C(O)NH-. In some cases, Z^4 is -O-. In some cases, Z^4 is -NH-.

In some embodiments of L^A, a is 1-4; b is 1-4; c is 1-3; d is 1-3; e is 1-6; and f is 1-3. In some embodiments, a is 4; b is 1; c is 2; d is 2; e is 5; and f is 2.

In some embodiments, Z^4 is -NHC(O)NH- and a is 1-4; b is 1-4; c is 1-3; r is 1; d is 1-3; e is 1-6; and f is 1-3. In some embodiments, Z^4 is -NHC(O)- and a is 1-4; b is 1-4; c is 1-3; r is 1; d is 1-3; e is 1-6; and f is 1-3.

In some embodiments, the linker (i.e., L^1-L^6) comprises L^B:



(L^B)

wherein:

a is 0 to 12 (e.g., 2 to 6, or 2, or 3);

b is 1 to 6 (e.g., 1, 2, or 3);

c is 1 to 6 (e.g., 1, 2, or 3);

r is 1 or 2;

d is 1 to 6 (e.g., 1, 2, or 3);

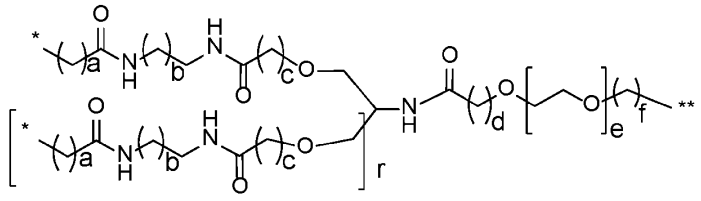
e is b is 1 to 6 (e.g., 1, 2, or 3); and

f is 1 to 6 (e.g., 1, 2, or 3).

In some embodiments of L^B, a is 1-4; b is 1-4; c is 1-3; r is 1; d is 1-3; e is 1-6; and f is 1-3. In some embodiments, a is 4; b is 1; c is 2; r is 1; d is 2; e is 5; and f is 2. In some embodiments, a is 2; b is 1; c is 2; r is 1; d is 2; e is 3; and f is 2. In some embodiments, a is 4; b is 1; c is 2; r is 1; d is 2; e is 3; and f is 2. In some embodiments, a is 1; b is 2; c is 2; r is 1; d is 2; e is 3; and f is 2. In some embodiments, a is 0; b is 3; c is 2; r is 1; d is 2; e is 3; and f is 2.

In some embodiments of L^B, a is 1-4; b is 1-4; c is 1-3; r is 2; d is 1-3; e is 1-6; and f is 1-3. In some embodiments, a is 2; b is 1; c is 2; r is 2; d is 2; e is 3; and f is 2. In some embodiments, a is 4; b is 1; c is 2; r is 2; d is 2; e is 3; and f is 2. In some embodiments, a is 1; b is 2; c is 2; r is 2; d is 2; e is 3; and f is 2. In some embodiments, a is 0; b is 3; c is 2; r is 2; d is 2; e is 3; and f is 2.

In some embodiments, the linker (i.e., L^1-L^6) comprises L^C:



(L^C)

wherein:

a is 0 to 12 (e.g., 1 to 6, 2 to 6, or 2, or 3);

b is 1 to 6 (e.g., 1 to 4, such as 1, 2, or 3);

c is 1 to 6 (e.g., 1 to 3, such as 1, 2, or 3);

r is 1 or 2;

d is 1 to 6 (e.g., 1 to 3, such as 1, 2, or 3);

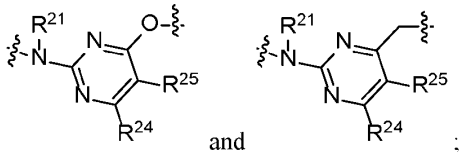
e is b is 1 to 6 (e.g., 1, 2, or 3); and

f is 1 to 6 (e.g., 1 to 3, such as 1, 2, or 3).

In some embodiments of L^c, a is 1-4; b is 1-4; c is 1-3; r is 1; d is 1-3; e is 1-6; and f is 1-3. In some embodiments, a is 2; b is 4; c is 2; r is 1; d is 2; e is 5; and f is 2.

In some embodiments of L^c, a is 1-4; b is 1-4; c is 1-3; r is 2; d is 1-3; e is 1-6; and f is 1-3. In some embodiments, a is 2; b is 4; c is 2; r is 2; d is 2; e is 5; and f is 2.

In some embodiments, the $-Z^1-L^1-$ includes a group selected from:



and

wherein R^{24} and R^{25} are each independently selected from H, optionally substituted $C_{(1-6)}$alkyl, optionally substituted fluoroalkyl, and halogen; and each R^{21} is independently selected from H, optionally substituted (C_1-C_6)alkyl, and optionally substituted alkanoyl. In some embodiments, R^{21} is H. In some embodiments, R^{24} is $C_{(1-3)}$-alkyl, or $C_{(1-3)}$-fluoroalkyl. In some cases, the fluoroalkyl is CF_3. In some embodiments, R^{25} is $C_{(1-3)}$-alkyl, or $C_{(1-3)}$-fluoroalkyl. In some cases, the fluoroalkyl is CF_3.

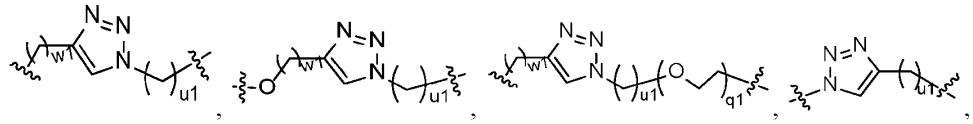
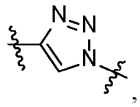
In some embodiments, the linker includes a polypeptide scaffold where some or all of the sidechain groups of the amino acid residues of such a polypeptide scaffold have been modified to attach a X binding moiety (e.g., as described herein). It is understood that X binding moieties (e.g., as described

herein) can be conjugated to amino acid residues, such as Asp, Lys, Orn, Glu, and Ser, of a polypeptide containing linker via a convenient conjugation chemistry. In some embodiments, the linker contains a polylysine polypeptide. In some embodiments, the linker contains a polyornithine polypeptide. In some embodiments, the linker contains a polyserine polypeptide. In some embodiments, the linker contains a polyaspartate polypeptide. The polypeptide backbone of such a linker can be a randomly polymerized polymer having an average length, or a polymer of defined length prepared e.g., in a controlled stepwise fashion. In some cases, the polypeptide linker has a length of 10-100 amino acid residues, such as 20-90, or 20-50 amino acid residues. In some embodiments, the N-terminal or C-terminal of the polypeptide linker is modified to include a linking moiety to an additional X binding moiety (e.g., as described herein). In some embodiments, the N-terminal or C-terminal of the polypeptide linker segment is modified with one or more linking moieties (e.g., as described herein) suitable for attachment to a protein construct (Y-B) including a polypeptide that specifically binds target autoantibody.

In some embodiments of the linker of formula (II), L^1 to L^3 each independently comprise one or more linking moieties independently selected from -C_{1-20}-alkylene-, -NHCO-C_{1-6}-alkylene-, -CONH-C_{1-6}-alkylene-, -NH C_{1-6}-alkylene-, -NHCONH-C_{1-6}-alkylene-, - NHCSNH-C_{1-6}-alkylene-, -C_{1-6}-alkylene-NHCO-, -C_{1-6}-alkylene-CONH-, -C_{1-6}-alkylene-NH-, -C_{1-6}-alkylene-NHCONH-, -C_{1-6}-alkylene-NHCSNH-, -$O(CH_2)_p$-, -$(OCH_2CH_2)_p$-, -NHCO-, -CONH-, -$NHSO_2$-, -SO_2NH-, -CO-, -SO_2-, -O-, -S-, monocyclic heteroaryl (e.g., 1,2,3-triazole), monocyclic aryl (e.g., phenyl, e.g., 1,4-linked phenyl or 1,3-linked phenyl), monocyclic heterocycle (e.g., pyrrolidine-2,5-dione, piperazine or piperidine ring as described herein), amino acid residue (naturally or non- naturally occurring amino acid residue), -NH-, and -NCH_3-, wherein each p is independently1 to 50.

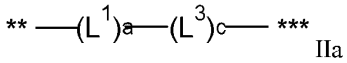
In some embodiments of the linker of formula (II), any of L^1-L^3 comprises repeating ethylene glycol moieties (e.g., -CH_2CH_2O- or -OCH_2CH_2-). In some embodiments, the linker of formula (II) comprises 1 to 25 ethylene glycol moieties, such as 3 to 25, 5 to 25, 7 to 25, 10 to 25, 15 to 25, 17 to 25, 20 to 25, or 22 to 25 ethylene glycol moieties. In some instances, the linker of formulae (II) comprises 3 or more ethylene glycol moieties, such as 5 or more, 7 or more, 10 or more, 15 or more, 20 or more, or even more ethylene glycol moieties.

In some embodiments of the linker of formula (II), any of L^1-L^3 comprises one or more triazole linking moieties. In some instances, the linker comprises one or more 1,2,3-triazole linking moieties. In some embodiments, the one or more 1,2,3-triazole moieties is selected from one of the following structures:

and , wherein w1, u1 and q1 are independently 1 to 25 (e.g., 1 to 12, such as 1 to 6).

In some embodiments of the linker of formula (II), n is 1, such that b is 0, and the linker is of the formula (IIa):



IIa

wherein

L^1 and L^3 are independently a linker (e.g., as described herein), wherein L^1 to L^3 together provide a linear linker between X and Y;

a is 1;

c is 0 or 1;

** represents the point of attachment to L^1 of X via Z^1; and

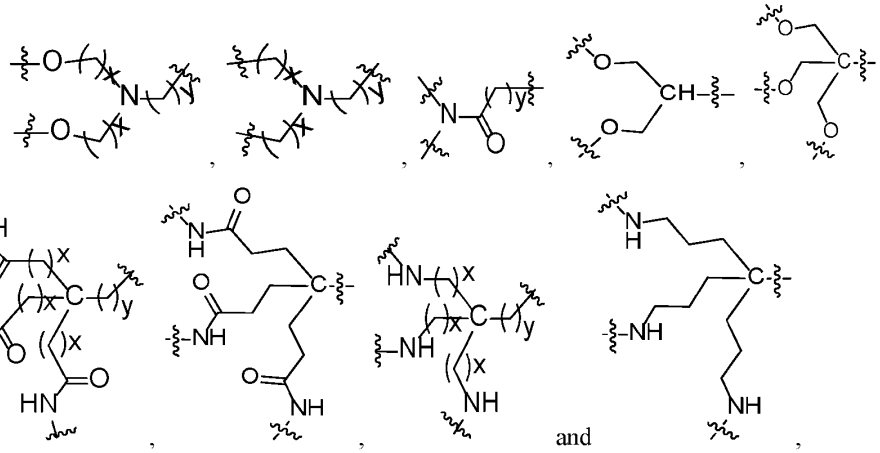
*** represents the point of attachment to Y.

In some embodiments, the linear linker has a backbone of 20 or more consecutive atoms covalently linking X to Y via Z^1, such as a backbone of 25 or more consecutive atoms, or 30 or more consecutive atoms, and in some cases, up to 100 consecutive atoms. In some embodiments of formula (IIa), the linear linker separates X and Y (or Z^1) by a chain of 20 to 50 consecutive atoms. In some embodiments, the linear linker separates X and Y (or Z^1) by a chain of 21 to 50 consecutive atoms, by a chain of 22 to 50 consecutive atoms, by a chain of 23 to 50 consecutive atoms, by a chain of 24 to 50 consecutive atoms, by a chain of 25 to 50 consecutive atoms, by a chain of 26 to 50 consecutive atoms, by a chain of 27 to 50 consecutive atoms, by a chain of 28 to 50 consecutive atoms, or by a chain of 29 to 50 consecutive atoms. In some embodiments of formula (IIa), the linear linker separates X and Y (or Z^1) by a chain of 30 to 60 consecutive atoms. In some embodiments, the linear linker separates X and Y (or Z^1) by a chain of 31 to 60 consecutive atoms. In some embodiments, the linear linker separates X and Y (or Z^1) by a chain of 32 to 60 consecutive atoms. In some embodiments, the linear linker separates X and Y (or Z^1) by a chain of 33 to 60 consecutive atoms. In some embodiments, the linear linker separates X and Y (or Z^1) by a chain of 34 to 60 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6)

comprises separates X and Y (or Z^1) by a chain of 35 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) comprises separates X and Y (or Z^1) by a chain of 36 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) comprises separates X and Y (or Z^1) by a chain of 41 to 50 consecutive atoms. In some embodiments, the linker (i.e., L^1-L^6) comprises separates X and Y (or Z^1) by a chain of 46 to 50 consecutive atoms.

In some embodiments, n is 2 or more, such that L^1 to L^3 together provide a branched linker between X and Y.

In some embodiments, n is 2 or more, and L^2 is selected from:



wherein each x and y are independently 1 to 10.

In some embodiments, L^1-L^2 comprises a backbone of 14 or more consecutive atoms between X and the branching atom, such as 14 to 50, 14 to 40, 14 to 35 or 14 to 30 consecutive atoms between X and the branching atom.

In some embodiments, L^3 comprises a backbone of 10 to 80 consecutive atoms, such as 12 to 70, 12 to 60, or 12 to 50 consecutive atoms.

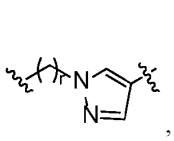
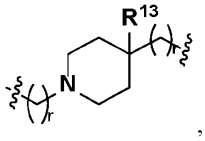
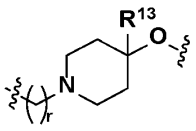
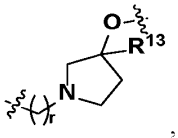
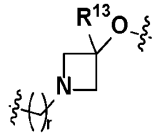
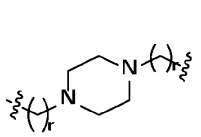
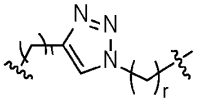
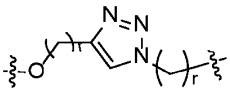
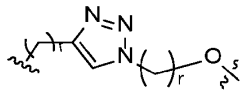
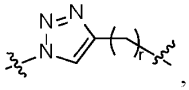
In some embodiments, wherein L^3 comprises a linking moiety selected from (C_{10}-C_{20}-alkylene (e.g., C_{12}-alkylene), or -$(OCH_2CH_2)_p$-, where p is 1 to 25, such as 3 to 25, 5 to 24, 7 to 25, 10 to 25, 15 to 25 or 20 to 24.

In some embodiments, L^1 to L^5 each independently comprise one or more linking moieties independently selected from -C_{1-20}-alkylene-, -NHCO-C_{1-6}-alkylene-, -CONH-C_{1-6}-alkylene-, -NH C_{1-6}-alkylene-, -NHCONH-C_{1-6}-alkylene-, - NHCSNH-C_{1-6}-alkylene-, -C_{1-6}-alkylene-NHCO-, -C_{1-6}-alkylene-CONH-, -C_{1-6}-alkylene-NH-, -C_{1-6}-alkylene-NHCONH-, -C_{1-6}-alkylene-NHCSNH-,

-$O(CH_2)_p$-, -$(OCH_2CH_2)_p$-, -NHCO-, -CONH-, -$NHSO_2$-, -SO_2NH-, -CO-, -SO_2-, -O-, -S-, monocyclic heteroaryl (e.g., 1,2,3-triazole), monocyclic aryl (e.g., phenyl, e.g., 1,4-linked phenyl or 1,3-linked phenyl), monocyclic heterocycle (e.g., pyrrolidine-2,5-dione, piperazine or piperidine ring as described herein), amino acid residue (naturally or non- naturally occurring amino acid residue), -NH-, and -NCH_3-, wherein each p is independently1 to 50.

In some embodiments, -$(L^1)_a$- comprises an optionally substituted alkyl or ethylene glycol linking moiety. In some embodiments, L^1 comprises an optionally substituted -C_{1-6}-alkylene-. In some embodiments, L^1 comprises an ethylene glycol linking moiety.

In some embodiments, each L^1 is independently selected from -C_{1-6}-alkylene-, -$(CH_2CH_2O)_t$-, -C_{1-6}-alkylene-NR^4CO-, -C_{1-6}-alkyleneCONH-, or OCH_2, wherein t is 1 to 20; and each R^4 is independently selected from H, and optionally substituted (C_1-C_6)alkyl. In some embodiments, each L^1 is -C_{1-6}-alkylene-, such as -C_{1-3}-alkylene-. In some embodiments, each L^1 is -$(CH_2CH_2O)_t$-, where t is 1 to 20, such as 1 to 15, 1 to 10, 1 to 8, 1 to 6, or 1 to 4. In some embodiments, each L^1 is --C_{1-6}-alkylene-NR^4CO-. In some embodiments, each L^1 is -C_{1-6}-alkyleneCONH-. In some embodiments, each L^1 is or OCH_2.

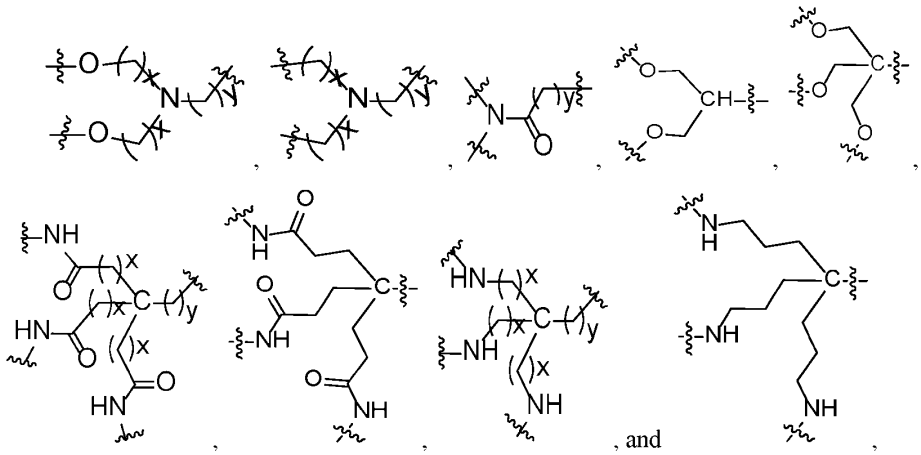
In some embodiments, one or more L^1 is independently -CH_2O-, -$(CH_2CH_2O)_t$-, -NR^4CO-, -C_{1-6}-alkylene-, , , , , , , , , , , or ,

wherein R^{13} is selected from H, halogen, OH, optionally substituted (C_1-C_6)alkyl, optionally substituted (C_1-C_6)alkoxy, COOH, NO_2, CN, NH_2, -$N(R^{21})_2$, -$OCOR^{21}$, -$COOR^{21}$, -$CONHR^{21}$, and -$NHCOR^{21}$; and each r independently 0 to 20, and any of the L^1 moieties are optionally further substituted.

In some embodiments, L^2 is -OCH_2-. In certain other embodiments, L^2 is $(OCH_2CH_2)_q$-, and q is 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3 or 1 to 2. In some embodiments, q is 2 to 8, such as 2 to 6 , 4 to 6, or 2 to 4.

In some embodiments, L^3 is absent or independently selected from -C_{1-6}-alkylene-, -$(CH_2CH_2O)_t$-, --C_{1-6}-alkylene-NHCO-, -C_{1-6}-alkyleneCONH-,or OCH_2, wherein t is 1 to 20. In some embodiments, L^3 is absent. In some embodiments, L^3 is -C_{1-6}-alkylene-. In some embodiments, L^3 is -$(CH_2CH_2O)_t$-, where t is 1 to 20, such as 1 to 15, 1 to 12, 1 to 10, 1 to 8, 1 to 6, 1 to 4 or 1 to 3. In some embodiments, L^3 is -C_{1-6}-alkylene-NHCO-. In some embodiments, L^3 is -C_{1-6}-alkyleneCONH-. In some embodiments, L^3 is OCH_2.

In some embodiments of formula (IIb), one or more L^4 is a branched linking moiety. In some embodiments of formula (IIb), one or more L^4 is a branched linking moiety selected from:



, and

wherein each x and y are each independently 1 to 10, such as 1-6, 1-3, e.g., 1 or 2. In In some embodiments, each x is 1, 2 or 3, e.g., 2.

In some embodiments, L^5 comprises one or more of: an amino acid residue (e.g., Asp, Lys, Orn, Glu), an amino acid analogue, N-substituted amido (-N(-)C(=O)-), tertiary amino, polyol (e.g., O-substituted glycerol), and the like. Analogs of an amino acid, include but not limited to, unnatural amino acids, as well as other modifications known in the art. The amino acid includes L-amino acids, D-amino acids, or both, and may contain any of a variety of amino acid modifications or analogs known in the art.

In some embodiments, the linker includes a polypeptide scaffold where some or all of the sidechain groups of the amino acid residues have been modified to attach a X binding moiety (e.g., as described herein). It is understood that X binding moieties (e.g., as described herein) can be conjugated to amino acid residues, such as Asp, Lys, Orn, Glu, and Ser, of a polypeptide containing linker via a convenient conjugation chemistry. In some embodiments, the linker contains a polylysine polypeptide. In some embodiments, the linker contains a polyornithine polypeptide. In some embodiments, the linker contains a polyserine polypeptide. In some embodiments, the linker contains a polyaspartate polypeptide.

The polypeptide can be a randomly polymerized polymer having an average length, or a polymer of defined length prepared e.g., in a controlled stepwise fashion. In some cases, the polypeptide linker segment has a length of 10-100 amino acid residues, such as 20-90, or 20-50 amino acid residues. In some embodiments, the N-terminal or C-terminal of the polypeptide linker segment is modified to include a linking unit to an additional M6PR binding moiety (e.g., as described herein). In some embodiments, the N-terminal or C-terminal of the polypeptide linker segment is modified with one or more linking units (e.g., as described herein) suitable for attachment to a Y moiety of interest.

In some embodiments, a is 1. In some embodiments, at least one of b, c, d, and e is not 0. In some embodiments, b is 1 or 2. In some embodiments, c is 1 or 2. In some embodiments, e is 1 or 2. In some embodiments, b, d and e are independently 1 or 2. In some embodiments, a, b, d, and e are each 1, and c is 0.

In some embodiments, the linker comprises 20 to 100 consecutive atoms, such as 20 to 90, 20 to 80, 20 to 70, 20 to 60, 20 to 50, 20 to 40 or 20 to 30 consecutive atoms. In some embodiments, the linker comprises 25 to 100 consecutive atoms, such as 30 to 100, 35 to 100, 40 to 100, 45 to 100, 50 to 100, 55 to 100, 60 to 100, 65 to 100, 70 to 100, 75 to 100, 80 to 100, 85 to 100, 90 to 100, or 95 to 100 consecutive atoms.

In some embodiments, the linker comprises 25 or more consecutive atoms, such as 26 or more, 27 or more, 28 or more, 29 or more or 30 or more consecutive atoms. In some embodiments, the linker comprises 30 or more consecutive atoms, such as 31 or more, 32 or more, 33 or more, 34 or more, 35 or more, 36 or more, 37, or more, 38 or more, 39 or more, 40 or even more consecutive atoms.

In some embodiments, the linker is a branched linker or linking moiety as shown in Table 1.

Table 1. Exemplary branched linkers and branched linking moieties

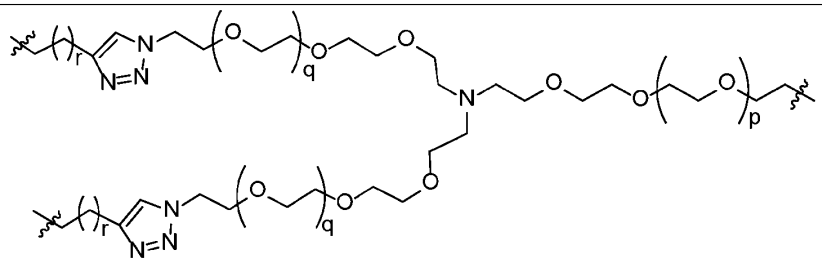
Linker No.	**Linker structure**
L21	 r is 0 to 10, q and p are independently 0 to 20

Table 1. Exemplary branched linkers and branched linking moieties

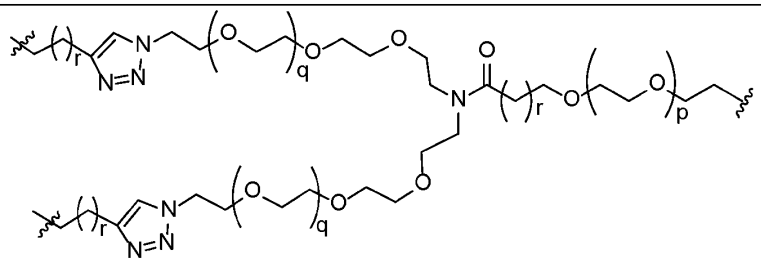
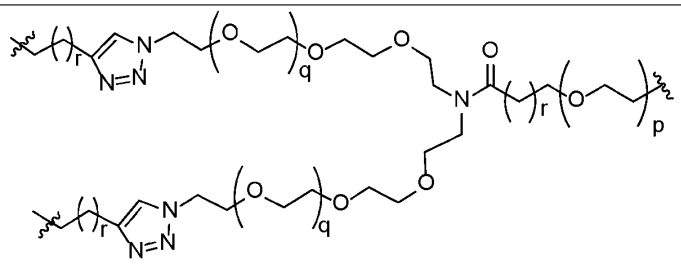
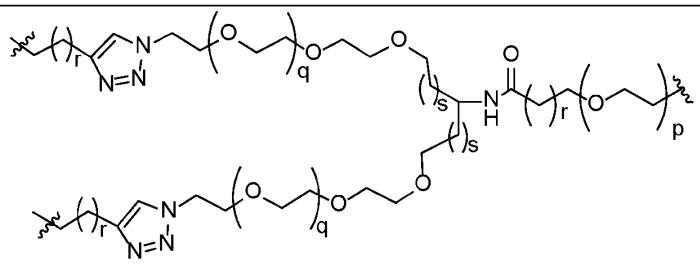
Linker No.	Linker structure
L22	 each r is independently 0 to 10, q and p are independently 0 to 20
L23	 each r is independently 0 to 10, q and p are independently 0 to 20
L24	 each r is independently 0 to 10, s is 0 or 1, q and p are independently 0 to 20

Table 1. Exemplary branched linkers and branched linking moieties

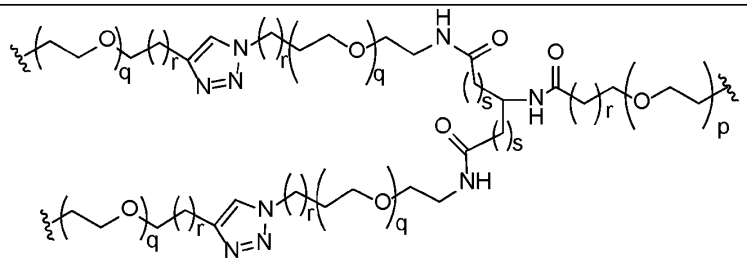
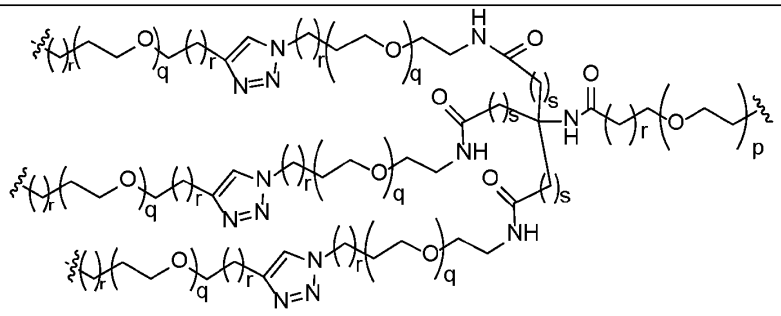
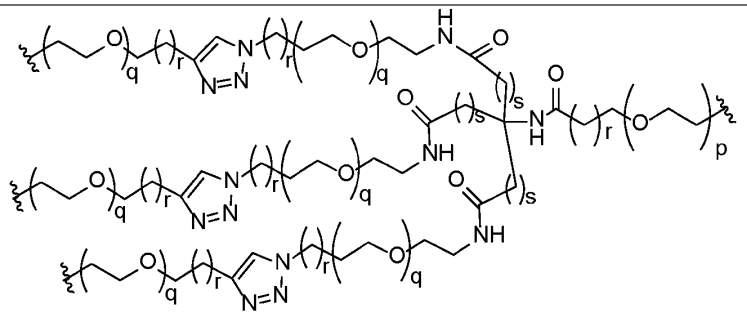
Linker No.	Linker structure
L25	 each r is independently 0 to 10, s is 0 or 1, each q and p is independently 0 to 20
L26	 each r is independently 0 to 10, s is 0 or 1, each q and p is independently 0 to 20
L26.1	 each r is independently 0 to 10, s is 0 or 1, each q and p is independently 0 to 20

Table 1. Exemplary branched linkers and branched linking moieties

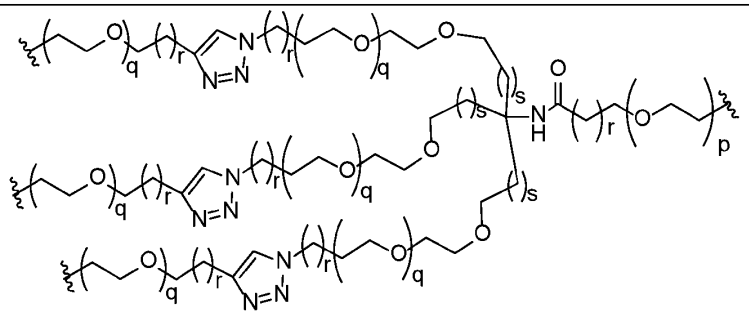
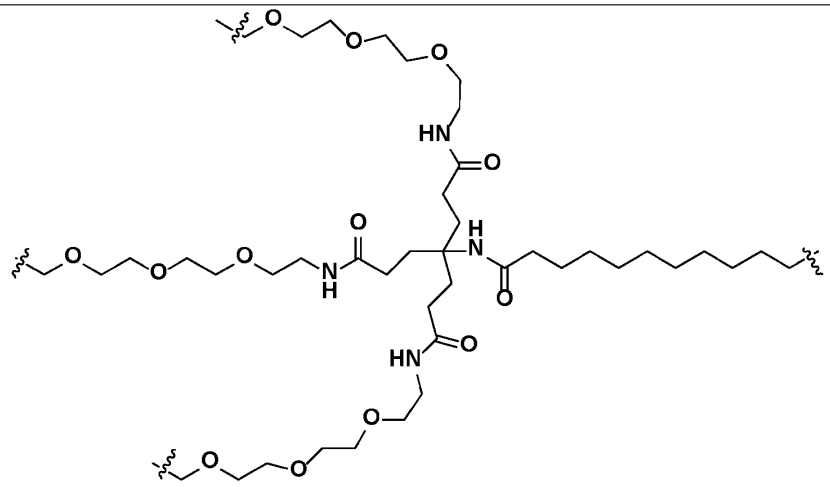
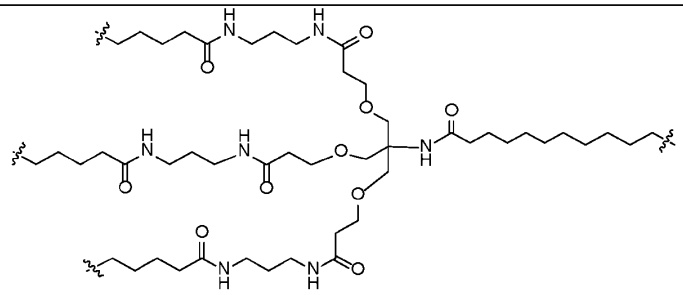
Linker No.	Linker structure
L27	 each r is independently 0 to 10, s is 0 or 1, each q and p is independently 0 to 20
L28	
L29	

Table 1. Exemplary branched linkers and branched linking moieties

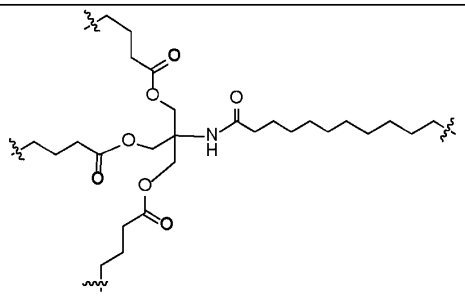
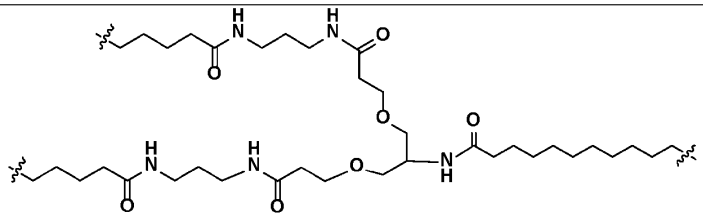
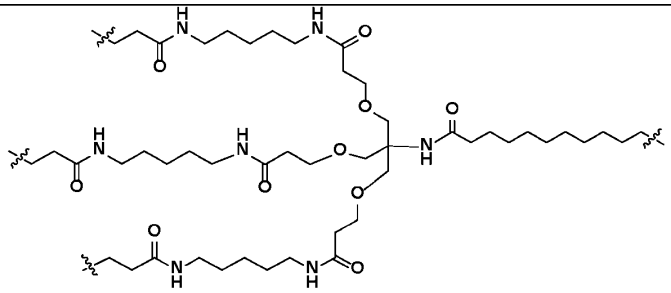
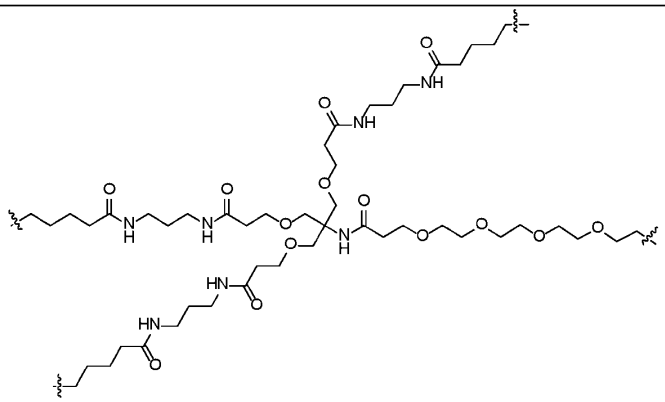
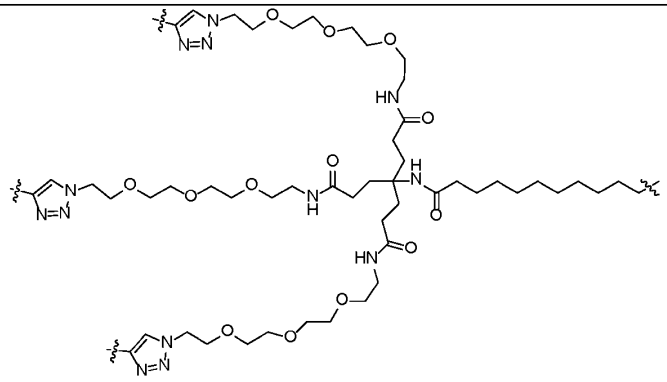
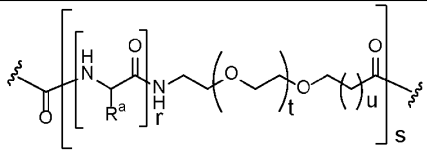
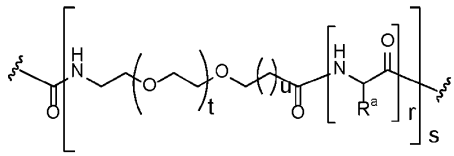
Linker No.	Linker structure
L30	
L31	
L32	
L33	

Table 1. Exemplary branched linkers and branched linking moieties

Linker No.	Linker structure
L34	
L35	
L36	
	where r is 1-3, t is 3-5, u is 0 or 1, and s is 2-5

A chemoselective ligation group is a group having a reactive functionality or function group capable of conjugation to a compatible group of a second moiety. For example, chemoselective ligation groups (or a precursor thereof) may be one of a pair of groups associated with a conjugation chemistry such as azido-alkyne click chemistry, copper free click chemistry, Staudinger ligation, tetrazine ligation, hydrazine-iso-Pictet-Spengler (HIPS) ligation, cysteine-reactive ligation chemistry (e.g., thiol-maleimide, thiol-haloacetamide or alkyne hydrothiolation), amine-active ester coupling, tyrosine specific conjugation chemistry (e.g., e-Y-CLICK), methionine specific conjugation chemistry (e.g., oxaziridine-based or ReACT chemistry), reductive amination, dialkyl squarate chemistry, etc.

Chemoselective ligation groups that may be utilized in linking two moieties, include, but are not limited to, amino (e.g., a N-terminal amino or a lysine sidechain group of a polypeptide), azido, aryl

azide, alkynyl (e.g., ethynyl or cyclooctyne or derivative), active ester (e.g., N-hydroxysuccinimide (NHS) ester, sulfo-NHS ester or PFP ester or thioester), haloacetamide (e.g., iodoacetamide or bromoacetamide), chloroacetyl, bromoacetyl, hydrazide, maleimide, vinyl sulfone, 2-sulfonyl pyridine, cyano-alkyne, thiol (e.g., a cysteine residue), disulfide or protected thiol, isocyanate, isothiocyanate, aldehyde, ketone, alkoxyamine, hydrazide, aminooxy, phosphine, HIPS hydrazinyl-indolyl group, or aza-HIPS hydrazinyl-pyrrolo-pyridinyl group, tetrazine, cyclooctene, squarate, and the like.

In some instances, chemoselective ligation group is capable of spontaneous conjugation to a compatible chemical group when the two groups come into contact under suitable conditions (e.g., copper free Click chemistry conditions). In some instances, the chemoselective ligation group is capable of conjugation to a compatible chemical group when the two groups come into contact in the presence of a catalyst or other reagent (e.g., copper catalyzed Click chemistry conditions).

In some embodiments, the chemoselective ligation group is a photoactive ligation group. For example, upon irradiation with ultraviolet light, a diazirine group can form reactive carbenes, which can insert into C-H, N-H, and O-H bonds of a second moiety.

In some instances, the terminal L moiety (e.g., the terminal L^6) is a precursor of the reactive functionality or function group capable of conjugation to a compatible group of a second moiety. For example, a carboxylic acid is a precursor of an active ester chemoselective ligation group.

In some embodiments, the terminal L moiety (e.g., the terminal L^6) is a reactive moiety capable forming a covalent bond to a polypeptide (e.g., with an amino acid sidechain of a polypeptide having a compatible reactive group). The reactive moiety can be referred to as a chemoselective ligation group.

In some embodiments, the terminal L moiety (e.g., the terminal L^6) is a thio-reactive chemoselective ligation group (e.g., as described in Table 2). In some cases, Y can produce a residual moiety Z resulting from the covalent linkage of a thiol-reactive chemoselective ligation group to one or more cysteine residue(s) of a protein, e.g., B or Y.

In some embodiments, the terminal L moiety (e.g., the terminal L^6) is a Cys-reactive chemoselective ligation group (e.g., a maleimide derivative as described herein). In some cases, the Cys-reactive chemoselective ligation group includes a maleimide group. In some embodiments, the chemoselective ligation group includes a maleimide group, e.g., mal-1 to mal-7.

In some embodiments, the terminal L moiety (e.g., the terminal L^6) is an amino-reactive chemoselective ligation group. In some cases, the terminal L moiety (e.g., the terminal L^6) can produce a residual moiety Z resulting from the covalent linkage of an amine-reactive chemoselective ligation group to one or more lysine residue(s) a protein, e.g., B or Y.

In some embodiments, the terminal L moiety (e.g., the terminal L^6) is a Lys-reactive chemoselective ligation group. In some embodiments the Lys-reactive chemoselective ligation group is a PFP ester.

Exemplary chemoselective ligation groups, and synthetic precursors thereof, which may be adapted for use in the compounds of this disclosure are shown in Table 2. In Table 2, the ~ can represent a point of attachment a linking moiety (or a linked X moiety).

Table 2: Exemplary chemoselective ligation groups and precursors

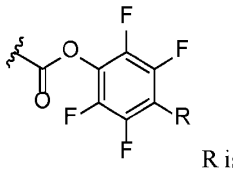
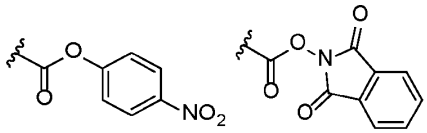
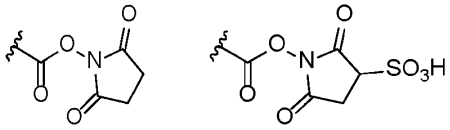
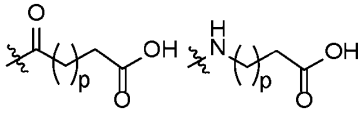
Groups	**Exemplary structures**
carboxylic acid or active ester	 where J is selected from -OH, -Cl, -Br, -I, -F, -OH, -O-N-succinimide, -O-(4-nitrophenyl), -O-pentafluorophenyl, -O-tetrafluorophenyl, and -O-C(O)-OR^{T}, and R^{T} is -C_1-C_8 alkyl or -aryl,  R is H or F,    ; where p is 0 to 6

Table 2: Exemplary chemoselective ligation groups and precursors

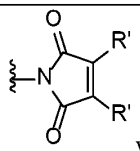
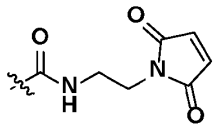
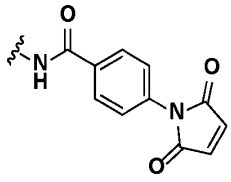
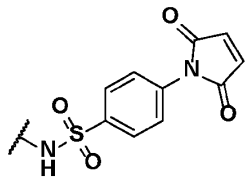
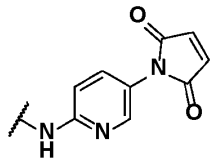
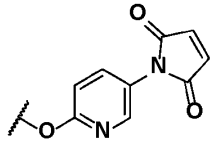
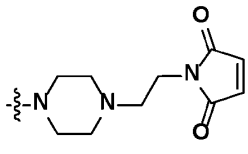
Groups	Exemplary structures
maleimide	 where each R' is independently hydrogen or halogen (e.g., bromo)  (mal-1)  (mal-2)  (mal-3)  (mal-4)  (mal-5)  (mal-6)

Table 2: Exemplary chemoselective ligation groups and precursors

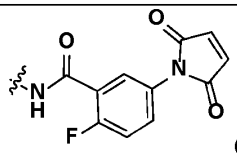
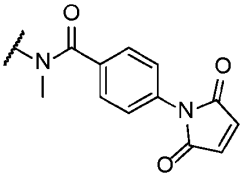
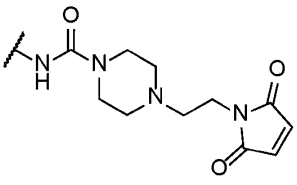
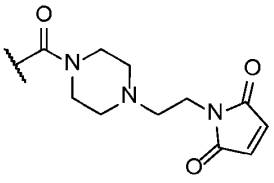
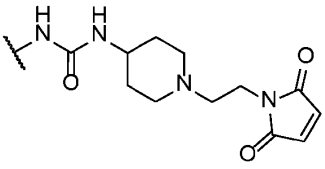
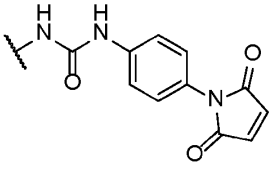
Groups	Exemplary structures
	 (mal-7)
	 (mal-8)
	 (mal-9)
	 (mal-10)
	 (mal-11)
	 (mal-12)

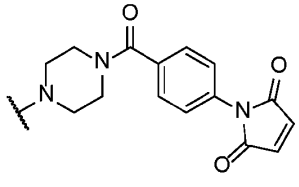
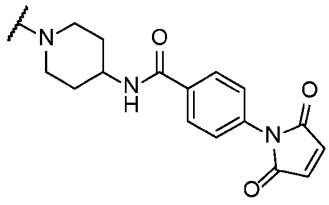
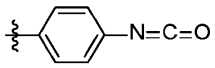
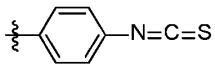
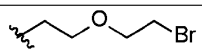
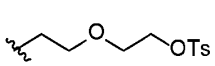
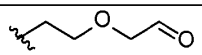
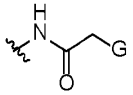
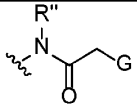
Table 2: Exemplary chemoselective ligation groups and precursors	
Groups	**Exemplary structures**
	 (mal-13)  (mal-14)
isocyanate or isothiocyanate	-NCS -NCO  
alkyl halide alkyl tosylate	 
aldehyde	
haloacetamide or alpha-leaving group acetamide	 where G is selected from -Cl, -Br, -I, -O-mesyl, and -O-tosyl
haloacetamide or alpha-leaving group acetamide	 where G is selected from -Cl, -Br, -I, -O-mesyl, and -O-tosyl; R'' is (C_1-C_6)alkyl,

Table 2: Exemplary chemoselective ligation groups and precursors

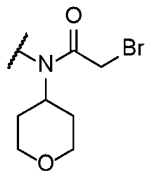
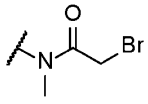
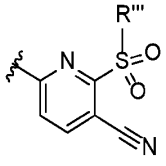
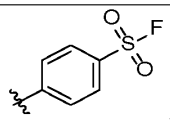
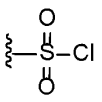
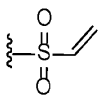
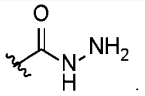
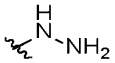
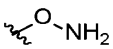
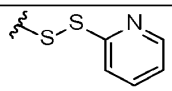
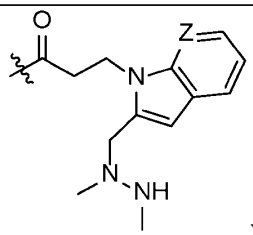
Groups	**Exemplary structures**
	heterocyclyl (e.g., 4-tetrahydro2H-pyran), or aryl  or 
2-sulfonylpyridine	 where R''' is alkyl
diazirine	
sulfonyl halide or vinyl sulfone	 ,  , or 
Hydrazide; hydrazine; hydroxylamino	 ;  ; 
pyridyl disulfide	
(HIPS) hydrazinyl-indolyl group, or (aza-HIPS) hydrazinyl-pyrrolo-pyridinyl group	 where Z is CH or N

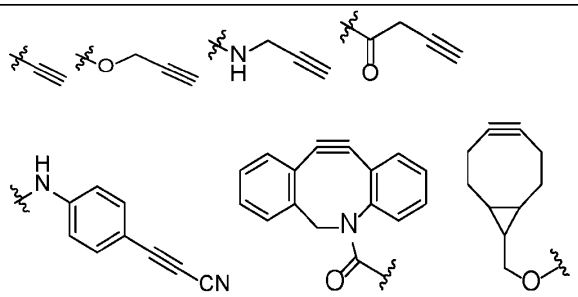
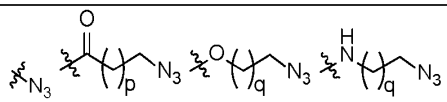
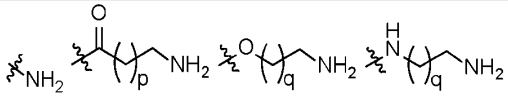
Table 2: Exemplary chemoselective ligation groups and precursors	
Groups	**Exemplary structures**
alkyne or cyclooctyne	
azide	 where p is 0 to 6 and where q is 1 to 6
amine	 where p is 0 to 6 and where q is 1 to 6

Table 3 shows exemplary residual moieties, wherein the "***" indicates the point of attachment of Y.

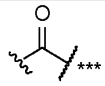
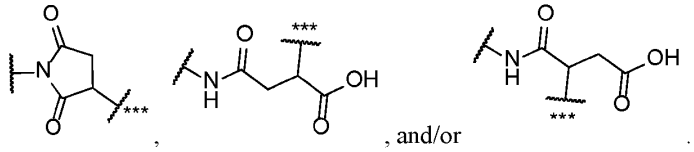
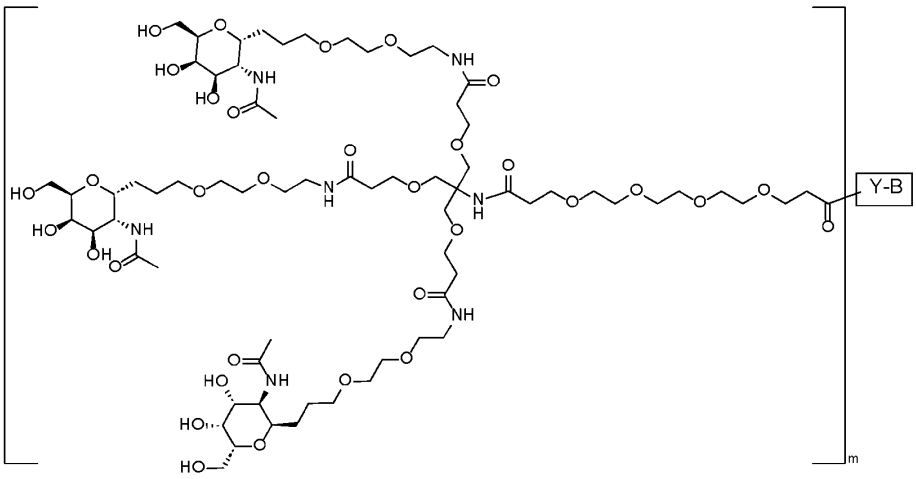
Table 3: Exemplary residual moieties from chemoselective ligation groups and precursors	
Groups	**Exemplary residual moieties**
carboxylic acid or active ester	
maleimide	
isocyanate or isothiocyanate	Urea or thiourea

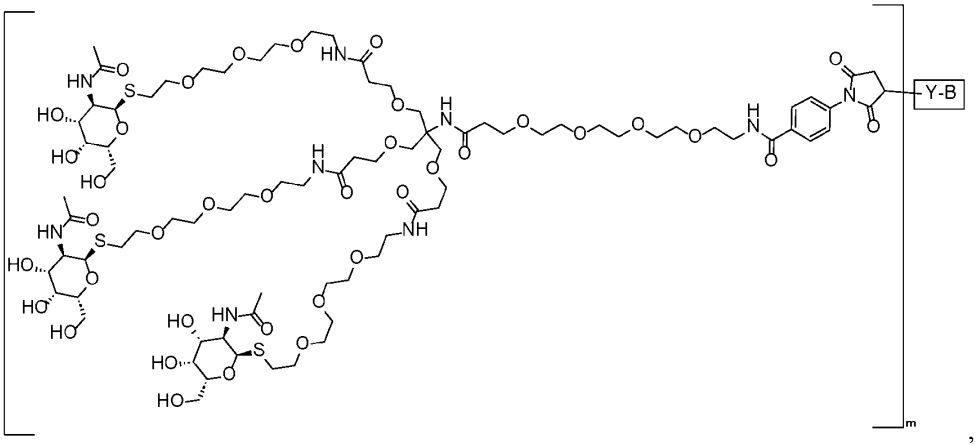
Table 3: Exemplary residual moieties from chemoselective ligation groups and precursors	
Groups	**Exemplary residual moieties**
alkyl halide alkyl tosylate	Direct bond
aldehyde	Imine
haloacetamide or alpha-leaving group acetamide	Direct bond

This disclosure includes compounds which can be prepared from a precursor ligand-linker compound including a chemoselective ligation group as described herein. The chemoselective ligation group of such compounds can be utilized to connect to a Y-B complex. It is understood that any of these compounds can also be prepared de novo to include an alternative Y moiety of interest (e.g., as described below) rather than the chemoselective ligation group. In some embodiments, such compounds are referred to as a conjugate, e.g., a biomolecule conjugate that specifically binds a target protein.

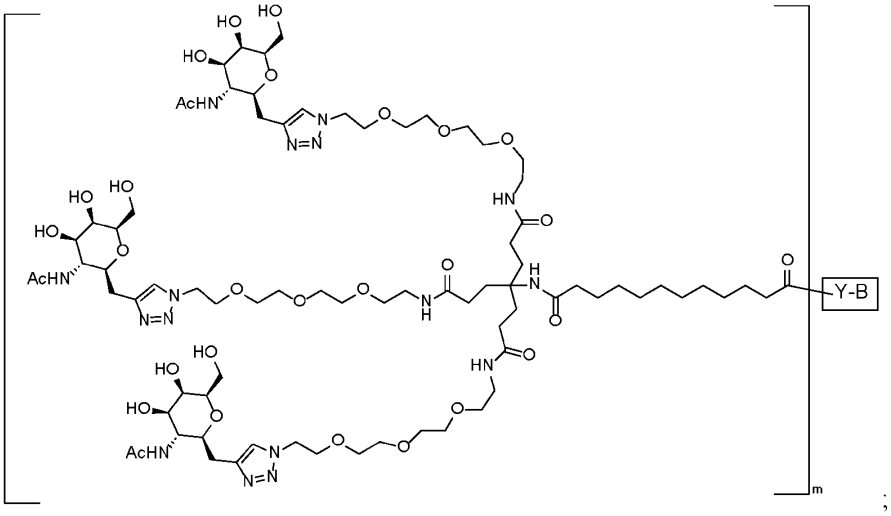
The examples described herein show specific, non-limiting, ASGPR binding compounds having chemoselective ligation group.

For example, in some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:


HO
HO
HO
NH
O
NH
O
O
O



, or



;

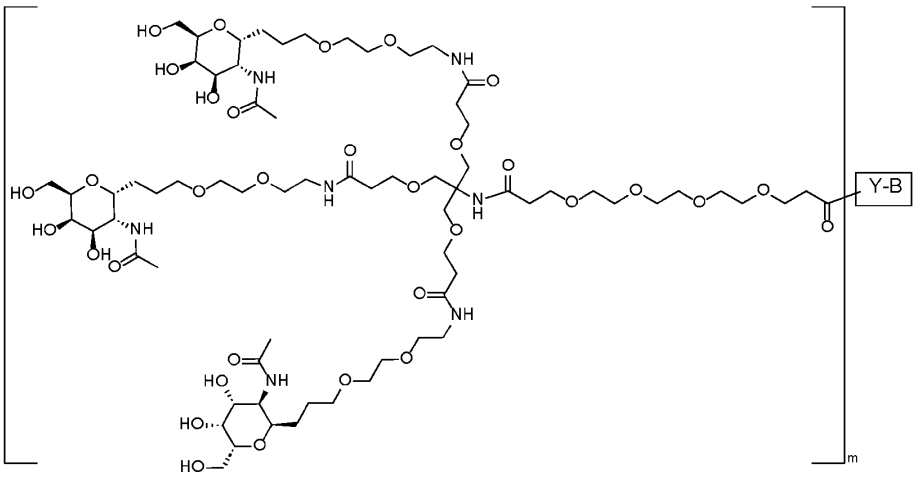
wherein:

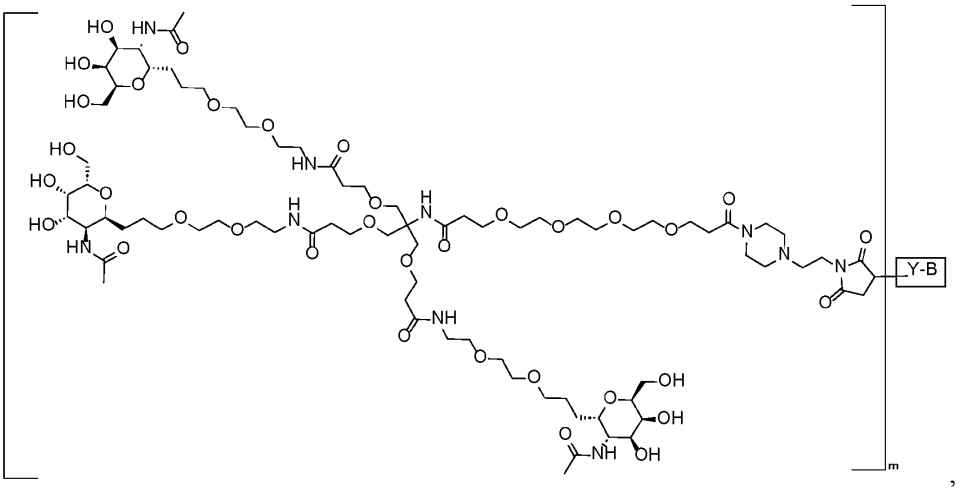
m is 1 to 10;

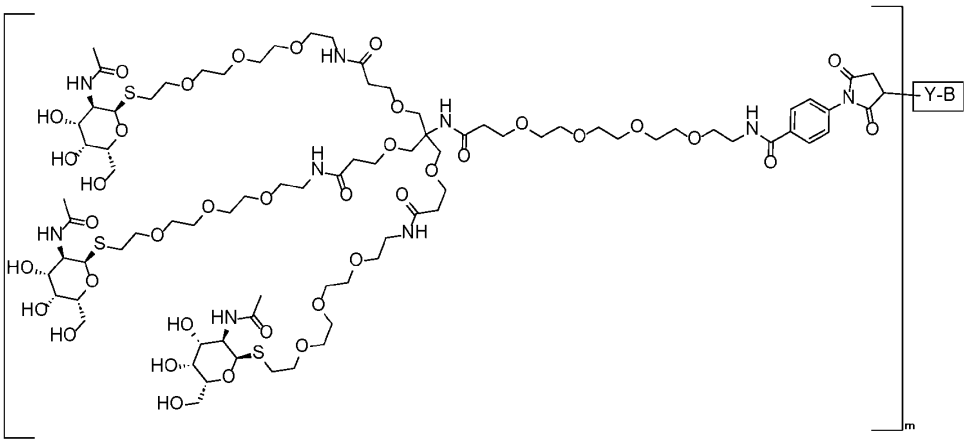
Y is a carrier polypeptide connected to B; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody.

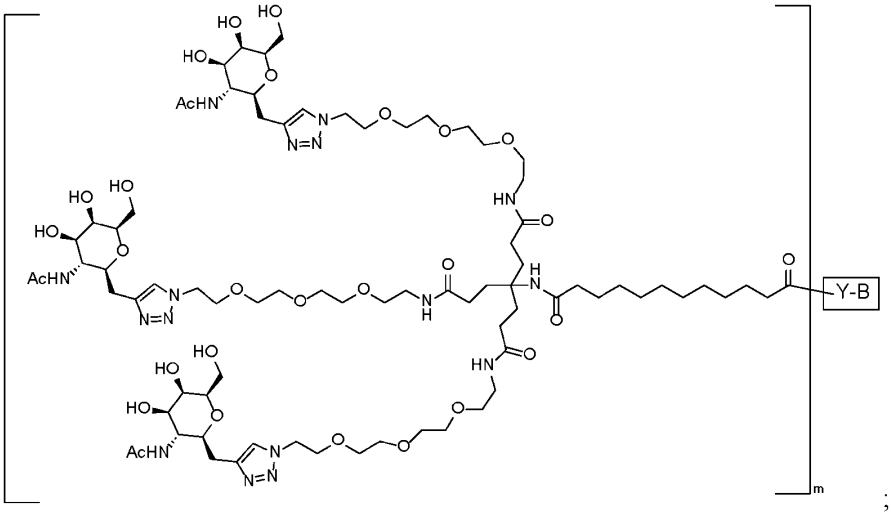
In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:


Y-B
m


Y-B
m



, or



;

wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody.

Exemplary carrier polypeptides and thyroid-stimulating hormone receptor (TSHR) polypeptides are described herein.

1.1.4 TSHR Polypeptides

As summarized above, the bifunctional compounds of this disclosure can include a thyroid stimulating hormone receptor (TSHR) polypeptide that specifically binds a target anti-TSHR autoantibody.

Thyroid stimulating hormone receptor is a postsynaptic integral membrane protein that plays a pivotal role in the development of the neuromuscular junction synapse (NMJ). Anti-TSHR autoantibodies are associated with Graves' disease. In some embodiments, the anti-TSHR autoantibody is an IgG antibody, e.g., an IgG2 antibody.

TSHR is an 87 kDa transmembrane receptor composed of a large amino-terminal extracellular domain (ECD) and a transmembrane domain (TMD). The TSHR ECD contains an N-terminal domain, a leucine-rich repeat domain (LRD) and a hinge region or cleavage domain. The TMD contains the typical seven transmembrane helices of GPCRs, an eighth helix parallel to the membrane and a C-terminal tail.

The extracellular domains (ECD) of the TSHR molecule contain the sites of binding (e.g., epitopes) of the disease-causing anti-TSHR autoantibodies (e.g., M22). In particular, the leucine-rich repeat domain (LRD) of TSHR can be considered antigens that contain the primary immunogenic or epitope-containing regions of the TSHR protein where anti-TSHR autoantibodies predominantly bind.

In some embodiments, the TSHR polypeptide is an antigen that includes one or more epitope-containing domains, or antibody binding fragments thereof, of the full length TSHR protein. In some embodiments, the TSHR polypeptide includes an extracellular domain of TSHR, or an antigenic (e.g., epitope-containing) fragment thereof. In some embodiments, the TSHR polypeptide includes leucine-rich repeat domain (LRD) of TSHR, or an antigenic fragment thereof.

In some embodiments, the TSHR polypeptide includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid addition, deletion, or substitution compared to a sequence of a human TSHR protein (SEQ ID NO: 1), or an antigenic fragment thereof. In some embodiments, the TSHR polypeptide has at least 80%, at least 85%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% sequence identity to the human TSHR protein (SEQ ID NO: 1) or an antigenic portion (e.g., antigenic domain or fragment thereof) of the human TSHR protein (SEQ ID NO: 1). In SEQ ID NO: 1 below (Table 4), the leucine-rich repeat domain (LRD) is bold and underlined, and the rest of extracellular domain is underlined.

In some embodiments the TSHR polypeptide is derived from the extracellular domain of TSHR, which is shown in Table 4 below. In some embodiments, the TSHR polypeptide includes 1, 2, 3, 4, 5, 6,

7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid addition, deletion, or substitution compared to a sequence of Table 4, or an antigenic fragment thereof. In some embodiment, the TSHR polypeptide includes a sequence having at least 80%, at least 85%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% sequence identity to a sequence of Table 4, or an antigenic fragment thereof. In some embodiments, the TSHR polypeptide includes a polypeptide of Table 4. In some embodiments, the TSHR polypeptide consists essentially of a polypeptide of one of Table 4.

Table 4: Exemplary TSHR polypeptide sequences

TSHR polypeptide	**Sequence**	**SEQ ID NO:**
Human TSHR	MRPADLLQLVLLLDLPRDLG**GMGCSSPPCECHQEEDFRVTCKDIQRIPSL PPSTQTLKLIETHLRTIPSHAFSNLPNISRIYVSIDVTLQQLESHSFYNL SKVTHIEIRNTRNLTYIDPDALKELPLLKFLGIFNTGLKMFPDLTKVYST DIFFILEITDNPYMTSIPVNAFQGLCNETLTLKLYNNGFTSVQGYAFNGT KLDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTALPSKGLEHLK ELIARN**TWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKIRGILESLM CNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNA HYYVFFEEQEDEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTP KSDEFNPCEDIMGYKFLRIVVWFVSLLALLGNVFVLLILLTSHYKLNVPR FLMCNLAFADFCMGMYLLLIASVDLYTHSEYYNHAIDWQTGPGCNTAGFF TVFASELSVYTLTVITLERWYAITFAMRLDRKIRLRHACAIMVGGWVCCF LLALLPLVGISSYAKVSICLPMDTETPLALAYIVFVLTLNIVAFVIVCCC YVKIYITVRNPQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILN KPLITVSNSKILLVLFYPLNSCANPFLYAIFTKAFQRDVFILLSKFGICK RQAQAYRGQRVPPKNSTDIQVQKVTHEMRQGLHNMEDVYELIENSHLTPK KQGQISEEYMQTVL	1
Extracellular Domain	MGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETHLRTIPSHA FSNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPDA LKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVNA FQGLCNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAF GGVYSGPSLLDVSQTSVTALPSKGLEHLKELIARNTWTL	2
Leucine-rich	GMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETHLRTIPSH	3

TSHR polypeptide	Sequence	SEQ ID NO:
repeat domain (LRD)	AFSNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPD ALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVN AFQGLCNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDA FGGVYSGPSLLDVSQTSVTALPSKGLEHLKELIARN	

In some embodiments, the TSHR polypeptide includes one or more amino acid substitutions selected from H63C, R112P, D143P, D151E, V169R, C176S, K250Q, and I253R as compared to a wild type TSHR (SEQ ID NO: 1). In some embodiments, the TSHR polypeptide includes one or more amino acid substitutions selected from R112P, D143P, D151E, V169R, C176S, K250Q, and I253R as compared to a sequence of SEQ ID NO: 1. In some embodiments, the TSHR polypeptide includes one or more amino acid substitutions selected from H63C, R112P, D143P, D151E, V169R, I253R as compared to a sequence of SEQ ID NO: 1. For example, the TSHR polypeptide includes a sequence having at least 80% ,at least 85%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% sequence identity to a sequence of Table 5, or an antigenic fragment thereof. In some embodiments, the TSHR polypeptide includes a polypeptide of Table 5. In some embodiments, the TSHR polypeptide consists essentially of a polypeptide of one of Table 5.

In some embodiments, the TSHR polypeptide, B of Formula I, comprises any one of SEQ ID NOs: 1-7. In some embodiments, the TSHR polypeptide, B of Formula I, comprises SEQ ID NO: 6. In some embodiments, Y-B comprises any one of SEQ ID NOs: 1-7. In some embodiments, the TSHR polypeptide, B of Formula I, includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid addition, deletion, or substitution compared to any one of SEQ ID NOs: 1-7, or an antigenic fragment thereof. In some embodiment, the TSHR polypeptide, B of Formula I, includes a sequence having at least 80%, at least 85%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 100% sequence identity to any one of SEQ ID NOs: 1-7, or an antigenic fragment thereof. In some embodiments, the TSHR polypeptide, B of Formula I, includes a polypeptide of any one of SEQ ID NOs: 1-7. In some embodiments, the TSHR polypeptide, B of Formula I, consists essentially of a polypeptide any one of SEQ ID NOs: 1-7.

Table 5: Exemplary TSHR polypeptide sequences

TSHR polypeptide	Sequence	SEQ ID NO:
Mutant TSHR polypeptide 1	GCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETCLRTIPSHAF SNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTPNLTYIDPDAL KELPLLKFLGIFNTGLKMFPPLTKVYSTEIFFILEITDNPYMTSIPRNAF QGLCNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFG GVYSGPSLLDVSQTSVTALPSKGLEHLKELRARNTWTL	4
Mutant TSHR polypeptide 3	MGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETCLRTIPSHA FSNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTPNLTYIDPDA LKELPLLKFLGIFNTGLKMFPPLTKVYSTEIFFILEITDNPYMTSIPRNA FQGLCNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAF GGVYSGPSLLDVSQTSVTALPSKGLEHLKELRARNTWTL	5
Mutant TSHR polypeptide 2	GCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETHLRTIPSHAF SNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTPNLTYIDPDAL KELPLLKFLGIFNTGLKMFPPLTKVYSTEIFFILEITDNPYMTSIPRNAF QGLSNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFG GVYSGPSLLDVSQTSVTALPSKGLEHLQELRARNTWTL	6
Mutant TSHR polypeptide 2	GCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETHLRTIPSHAF SNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTPNLTYIDPDAL KELPLLKFLGIFNTGLKMFPPLTKVYSTEIFFILEITDNPYMTSIPRNAF QGLSNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFG GVYSGPSLLDVSQTSVTALPSKGLEHLKELRARNTWTL	7

1.1.5 Carrier Polypeptide

A number of strategies, such as attachment of a carrier, for imparting desirable pharmacokinetic properties to a peptide or protein (e.g., avoidance of rapid renal clearance, and/or optimizing Fc receptor mediated recycling) can be adapted for use in the bifunctional molecules of this disclosure that contain a target binding the TSHR polypeptide.

The TSHR polypeptide can be connected to a carrier polypeptide Y that imparts one or more desirable properties onto the resulting bifunctional molecule (e.g., increase *in vivo* stability and/or half-

life), and/or provides for conjugation sites to a ligand-linker moiety, e.g., without disrupting the autoantibody binding properties of the TSHR polypeptide.

In some embodiments, the TSHR polypeptide is synthetically linked to a carrier polypeptide Y. In some embodiments of formula (I), the carrier polypeptide Y is conjugated to a TSHR polypeptide (B) via a bifunctional linker.

In some embodiments, the TSHR polypeptide is linked to a carrier polypeptide Y as part of a protein construct, such as a chimeric fusion protein. In some embodiments, the TSHR polypeptide B and the carrier polypeptide Y are fused directly to each other, e.g., via N-terminal to C-terminal fusion, or C-terminal to N-terminal fusion. In some embodiments, the TSHR polypeptide B and the carrier polypeptide Y are fused indirectly via a spacer domain. In some embodiments, the Y-B of formula (I) is a chimeric protein having a polypeptide encoded by a nucleic acid molecule that encodes for both a TSHR polypeptide B and the carrier polypeptide Y.

The carrier polypeptide (Y) can be a serum protein or domain of a serum protein. In some embodiments, the serum protein is an albumin. In some embodiments, the serum protein is an immunoglobulin. In some embodiments, the carrier polypeptide (Y) is an engineered serum protein or domain thereof. The carrier polypeptide (Y) can be prepared synthetically, or recombinantly.

1.1.5.1 Albumin

The carrier polypeptide can be a serum albumin protein or domain or subdomain thereof, or fragment thereof. In some embodiments, the carrier polypeptide is human serum albumin (HSA).

Albumins generally have a long plasma half-life (e.g., about 3 weeks) and can provide a scaffold to which bioactive molecules can be attached or fused. HSA has a long serum half-life in humans that is attributed in part to its interaction with neonatal Fc receptor (FcRn). HSA (molecular mass 66.5 kDa) includes three structurally similar and flexible domains: I (residues 1-195), II (196-383) and III (384-585). Each domain (D) is composed of two subdomains A and B (e.g., DIA [5-105], DIB [119-195], DIIA [196-292], DIIB [314-383], DIIIA [384-491], DIIIB [510-582], e.g., as referenced to SEQ ID NO: 2 of WO2017/029407) with common structural motifs.

In some embodiments, the carrier protein is an HSA variant having enhanced FcRn binding. FcRn binds to the C-terminal end of DIII of HSA and protects albumin from intracellular degradation. For instance, a single amino acid substitution within HSA DIII (K537P) shows 12-fold improved binding affinity to FcRn, which translates into longer half-life. Various amino acid residues of albumin located in Domain I or Domain II also affect HSA interaction with FcRn (e.g., WO 2013/135896 describes albumin

variants having one or more alterations in Domain I and one or more alterations in Domain III; WO 2015/036579 describes albumin variants having one or more alterations in Domain II).

Any convenient albumin protein can be a parent for an albumin variant that can be utilized as a carrier polypeptide. As an example, human serum albumin (HSA) such as AAA98797, P02768-1, SEQ ID NO. 15 (mature HSA), or SEQ ID NO. 14 (precursor HSA).

In some embodiments, other albumins are utilized as a carrier polypeptide. Such other albumins include, but are not limited to, primate serum albumin (e.g.,, chimpanzee serum albumin, XP_517233.2), gorilla serum albumin or macaque serum albumin (e.g., NP_001 182578), rodent serum albumin (e.g.,, hamster serum albumin, A6YF56), guinea pig serum albumin (e.g., Q6WDN9-1), mouse serum albumin (e.g., AAH49971, P07724-1 Version 3) and rat serum albumin (e.g., AAH85359, P02770-1 Version 2), bovine serum albumin (e.g., cow serum albumin P02769-1), equine serum albumin such as horse serum albumin (e.g., P35747-1), or donkey serum albumin (e.g., Q5XLE4-1), rabbit serum albumin (e.g., P49065-1 Version 2), goat serum albumin (e.g., ACF10391), sheep serum albumin (e.g., P14639-1), dog serum albumin (e.g., P49822-1), chicken serum albumin (e.g., P19121 -1 Version 2) and pig serum albumin (e.g., P08835- 1 Version 2) or a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.2%, 99.4%, 99.6%, or at least 99.8% amino acid sequence identity to such an albumin. Non-mammalian albumins of interest include ovalbumin (e.g., P01012.pro: chicken ovalbumin; 073860. Pro: turkey ovalbumin).

In some embodiments, albumin, a fragment thereof, or conjugation-competent albumin variant, or albumin part of a fusion polypeptide or conjugate comprising albumin or a fragment thereof has a polypeptide sequence having at least 80%, 85%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 14 (e.g., P02768-1) below, where Domain I is in bold, Domain II is underlined, and Domain III is in italic (Table 6).

In some embodiments, albumin, a fragment thereof, or conjugation-competent albumin variant, or albumin part of a fusion polypeptide or conjugate comprising albumin or a fragment thereof has a polypeptide sequence having at least 80%, 85%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 15.

In some embodiments, the albumin, a fragment thereof, or conjugation-competent albumin variant, or variant thereof of the bifunctional molecule has a sequence identity to the sequence of HSA shown in SEQ ID NO: 15 of at least 80%, 85%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the albumin maintains at least one of the major properties of albumin or a similar tertiary structure as an albumin, such as HSA. A functional fragment of albumin may

have a sequence identity of at least 60%, 70%, 80%, 85%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.2%, 99.4%, 99.6%, 99.8%, or 100% to the sequence of HSA Domain III as shown in SEQ ID NO: 8, or to the sequence of HSA Domain II and Domain III as shown in SEQ ID NO: 9, or to a molecule consisting of or comprising two copies of Domain III (e.g., SEQ ID NO: 10), or to a molecule consisting of or comprising three copies of Domain III (e.g., SEQ ID NO: 11), or to a molecule consisting of or comprising Domain I and two copies of Domain III (e.g., SEQ ID NO: 12).

In some embodiments, the carrier polypeptide, or Y of Formula I, comprises any one of SEQ ID NOs: 8, 9, 10, 11, 12, 14, and 15. In some embodiments, Y comprises SEQ ID NO: 15.

Table 6

Name	**Sequence**	**SEQ ID NO:**
HSA	MKWVTFISLLFLFSSAYSRGVFRR**DAHKSEVAHRFKDLGEENFKALVLIAFAQ YLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRE TYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFL KKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDE GKASSAK**QRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKV HTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVE NDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLL RLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEE*PQNLIKQNCELFEQLGE YKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYL SVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAET FTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCC KADDKETCFAEEGKKLVAASQAA**LGL	14

Name	Sequence	SEQ ID NO:
HSA mature protein	DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTC VADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHK DDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAK RYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFK AWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYIC ENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKN YAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYA KVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLV EVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCC TESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALV ELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAAL GL	15
HSA Domain III	VEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKV GSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRP CFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKA TKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL	8
HSA Domain II and Domain III	DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTC VADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHK DDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAK RYKAAFTECCQAADKAACLLPKLDELRDEGKASSAVEEPQNLIKQNCELFEQL GEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAED YLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNA ETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEK CCKADDKETCFAEEGKKLVAASQAALGL	9

Name	Sequence	SEQ ID NO:
HSA 2 copies Domain III	VEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKV GSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRP CFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKA TKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGLVEEPQNL IKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKH PEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEV DETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKA VMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL	10
HSA 3 copies Domain III	VEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKV GSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRP CFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKA TKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGLVEEPQNL IKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKH PEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEV DETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKA VMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGLVEEPQNLIKQNCEL FEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMP CAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPK EFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA FVEKCCKADDKETCFAEEGKKLVAASQAALGL	11

Name	Sequence	SEQ ID NO:
HSA Domain I and 2 copies Domain III	DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTC VADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHK DDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAK RYKAAFTECCQAADKAACLLPKLDELRDEGKASSAVEEPQNLIKQNCELFEQL GEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAED YLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNA ETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEK CCKADDKETCFAEEGKKLVAASQAALGLVEEPQNLIKQNCELFEQLGEYKFQN ALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLN QLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHA DICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDK ETCFAEEGKKLVAASQAALGL	12

The human serum albumin (HSA) polypeptide chain has 35 cysteine residues, which form 17 disulfide bonds and one unpaired (free) cysteine at position 34 located in Domain I of the mature protein. The albumin, a fragment thereof, or conjugation-competent albumin variant, or albumin part of a fusion polypeptide or conjugate comprising albumin or a fragment thereof according to the disclosure, when folded, may have several, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and suitably all 17, of the native disulfide bonds of the polypeptide of SEQ ID NO. 15.

Albumin carrier polypeptides which can be adapted for use in the bifunctional molecules of this disclosure are described in WO 2017/029407, WO 2013/135896, WO 2015/036579, the disclosures of which are herein incorporated by reference in their entireties.

Conjugation of molecules to the free thiol on cysteine-34 of HSA can provide site-selective conjugation. To increase loading potential, recombinant HSA (rHSA) variants can be engineered to contain additional free, conjugation-competent cysteines.

The term "thio-albumin" is used to describe an albumin variant which comprises one or more (e.g. several) unpaired cysteine residues, particularly an albumin variant in which one or more (e.g. several) of the unpaired cysteine residues does not occur in a naturally occurring variant of an albumin. A thio-albumin refers to a conjugation-competent albumin. The rHSA variant can be a thio-albumin or a conjugation-competent albumin. The rHSA may have one or more free, conjugation-competent cysteines introduced in Domain I (DI), Domain II (DII), and/or Domain III (DIII). In some embodiments, the rHSA

variant has at least one free, conjugation-competent cysteines introduced in DI or DII. In some embodiments, the rHSA variant has at least one free, conjugation-competent cysteine introduced in DI and DIII. In some embodiments, the rHSA variant has a conjugation site at Cys34 and at least one or more free, conjugation-competent cysteines introduced in DI, DII, and/or DIII.

In some embodiments, the carrier polypeptide is an HSA variant engineered for increased stability, conjugation efficiency (Cys or Lys), and/or FcRn binding.

In some embodiments, the carrier polypeptide is a rHSA variant having a conjugation position at Cys34 (e.g., in reference to SEQ ID NO: 2) and one or more conjugation-competent cysteines introduced at positions selected from K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO: 15. The free, conjugation-competent cysteines may be introduced by substitution of an amino acid other than cysteine at any one of positions corresponding to or equivalent to any of residues selected from K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO. 15, or by insertion of a cysteine at a position adjacent to N- or C- side of an amino acid corresponding to a position equivalent to any of residues selected from K93, A226, E230, I271, E294, E358, L24, F49, V54, D56, A92, Q94, E97, H128, F156, E227, D237, K240, D259, K262, N267, Q268, L275, L284, K317, A322, E333, D340, E354, K359, A362, E382, and L398 of SEQ ID NO: 15. In some embodiments, at least one or more free, conjugation-competent cysteines is introduced at one or more positions corresponding to or equivalent to any of residues selected from C34, V54, H128, K240, and K262.

In some embodiments, the carrier polypeptide is a rHSA variant that exhibits up to 95% monomeric stability and retains hFcRn engagement compared with a wildtype unconjugated control (e.g., as demonstrated by Biolayer Interferometry). In some embodiments, introduction of the free, conjugation-competent cysteines into the rHSA variant does not interfere with FcRn binding. In some embodiments, introduction of the free, conjugation-competent cysteines maintains the albumin half-life in circulating blood.

1.1.5.2 Immunoglobulin

In some embodiments, the carrier polypeptide is an Fc fragment, a monomer, a dimer, a domain, or fragments thereof. Fc fragments contain the CH2 and CH3 domains and part of the hinge region held together by one or more disulfides and noncovalent interactions. Fc and Fc5μ fragments are produced from fragmentation of IgG and IgM, respectively. Fc fragments are derived from the heavy chain constant

region of an immunoglobulin. In some embodiments, Fc fragments are derived from IgG immunoglobulins of any subclass (e.g., IgG1, IgG2, IgG3, IgG4). In some embodiments, Fc fragments are derived from human IgG1.

The term "Fc dimer" refers to an Fc fragment containing two CH2-CH3 chains. The dimer is typically about 54k Da. An "Fc monomer" is generally half the size of the dimer (e.g., about 27k Da). Methods for generating Fc dimers and monomers are described in Wang et al. Engineering soluble monomeric IgG1 Fc with significantly decreased non-specific binding. Front. Immunol. (2017) 8:1545; Ying et al. Soluble monomeric IgG1 Fc. J. Biol Chem (2012) 287(23): 19399-408, each of which is incorporated herein by reference in its entirety.

In some embodiments, the Fc fragment has a polypeptide sequence having at least 80%, 85%, 90%, 91 %, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 13 (human IgG1 hinge-Fc) below:

DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF

NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT

ISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP

PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 13).

In some embodiments, the Fc fragment is a variant of a human IgG Fc region. In some embodiments, the Fc fragment is a variant of human IgG Fc comprising one or more amino acid substitutions in the CH2 and/or CH3 domains.

In some embodiments, the carrier polypeptide (Y) is an Fc fragment generated from the heavy chain constant region of an immunoglobulin, an Fc monomer, an Fc dimer, or fragments thereof. In some embodiments, the carrier polypeptide (Y) Fc is conjugated to an antibody fragment comprising an antigen-binding region (e.g., nanobody, scFv, VHH) to form an engineered antibody. In some embodiments, the carrier polypeptide (Y) Fc is conjugated to an antigen to form an Fc-antigen fusion. In various embodiments, the Fc fragment is conjugated to polypeptide B at a cysteine residue on the Fc fragment.

1.1.6 Peptide-Peptide Linker

In some embodiments, the carrier polypeptide (i.e., Y of Formula I) and the TSHR polypeptides (i.e., B of Formula I) are connected to B via a linker or linking sequence. In some embodiments, the linker is non-peptidic linking moiety. In some embodiments, the carrier polypeptide is fused directly to the TSHR polypeptide. In some embodiment, the carrier polypeptide is fused indirectly to B via, for example,

a spacer domain. In some embodiment, the N-terminal of the carrier polypeptide is fused to the C-terminal of the TSHR polypeptide. In some embodiment, the C-terminal of the carrier polypeptide is fused to the N-terminal of the TSHR polypeptide.

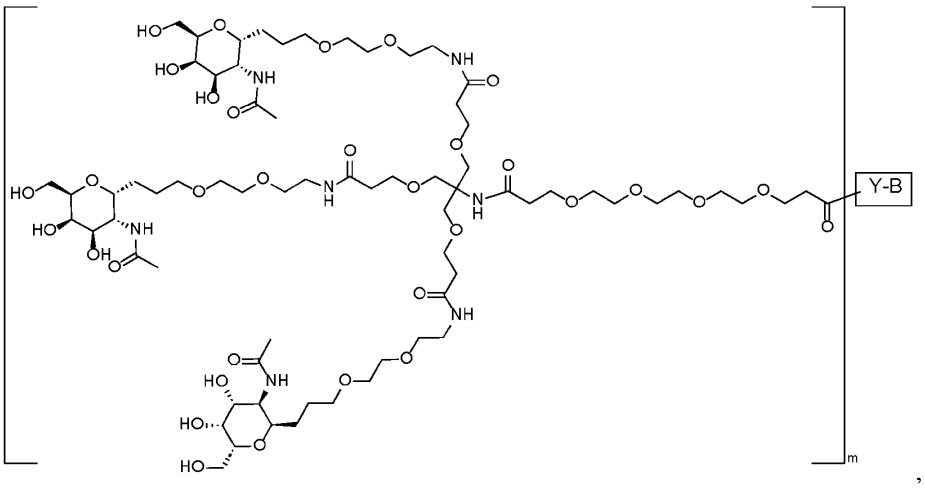
In some embodiments, the carrier polypeptide and the TSHR polypeptide may be connected via a linking sequence including one or more peptides. In some embodiments, the linking sequence may be polypeptides having 5 to 30 peptides. For example, the linking sequences may include $(GGGGS)_x$, wherein x is about 1 to about 10. In some embodiments, the linking sequence may include GGGGS (SEQ ID NO: 41), GGGGSGGGGS (SEQ ID NO: 42), GGGGSGGGGSGGGGS (SEQ ID NO: 16), or GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 43). In some embodiments, the linking sequence may consist essentially of one of SEQ ID NOs: 16, 41-43. In some embodiments, the linking sequence may include SEQ ID NO: 16. In some embodiments, the linking sequence may consist essentially of SEQ ID NO: 16.

1.1.7 Exemplary LYTACs

The examples described herein show specific, non-limiting, ASGPR binding compounds having chemoselective ligation group.

For example, in some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



,

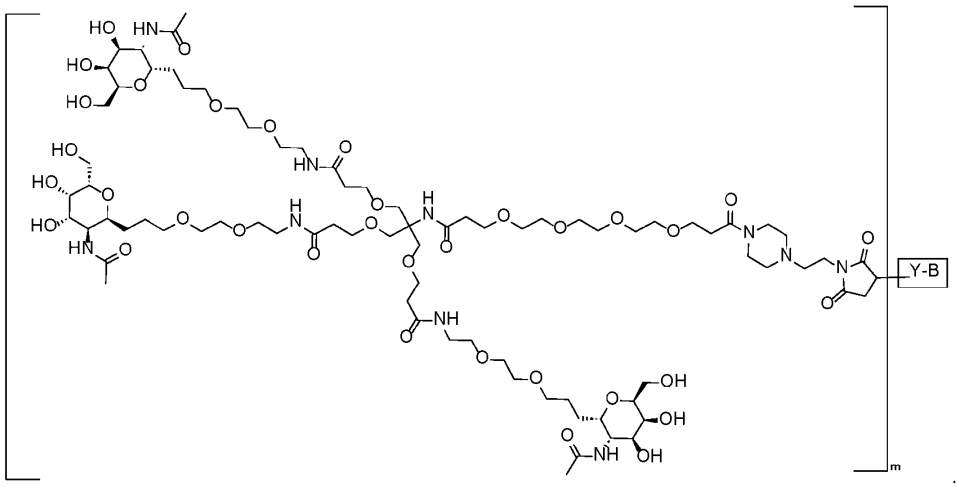
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, the TSHR polypeptide comprising SEQ ID NO: 6.

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



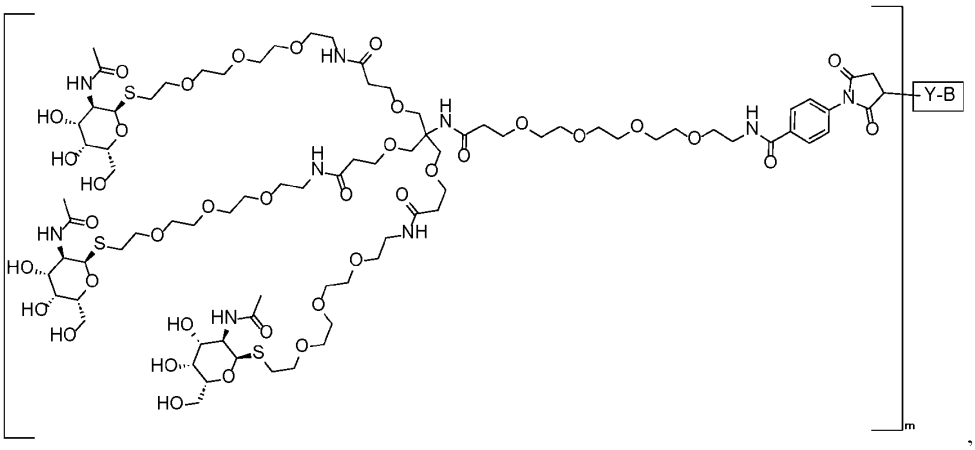
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, the TSHR polypeptide comprising SEQ ID NO: 6.

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



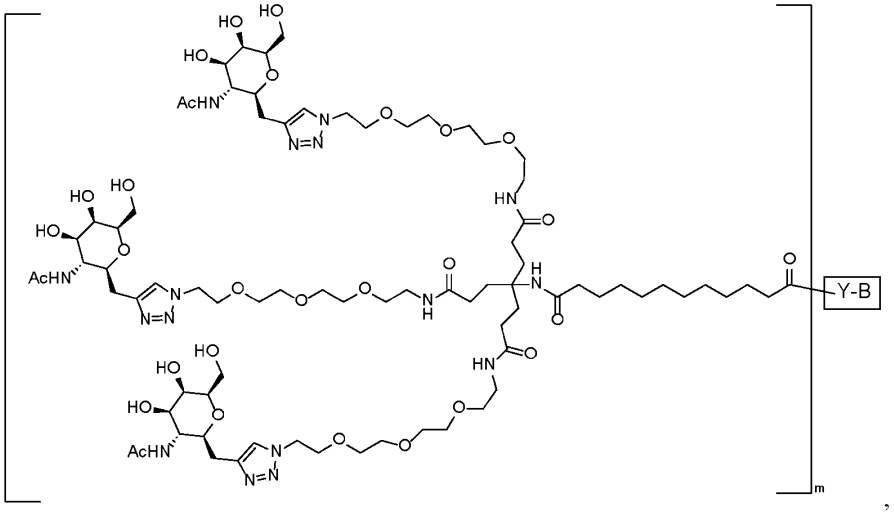
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, the TSHR polypeptide comprising SEQ ID NO: 6.

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



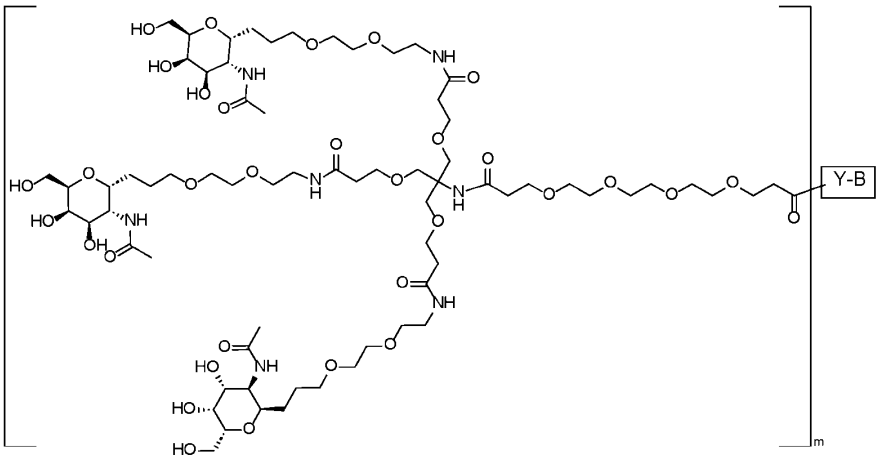
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, the TSHR polypeptide comprising SEQ ID NO: 6.

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



wherein:

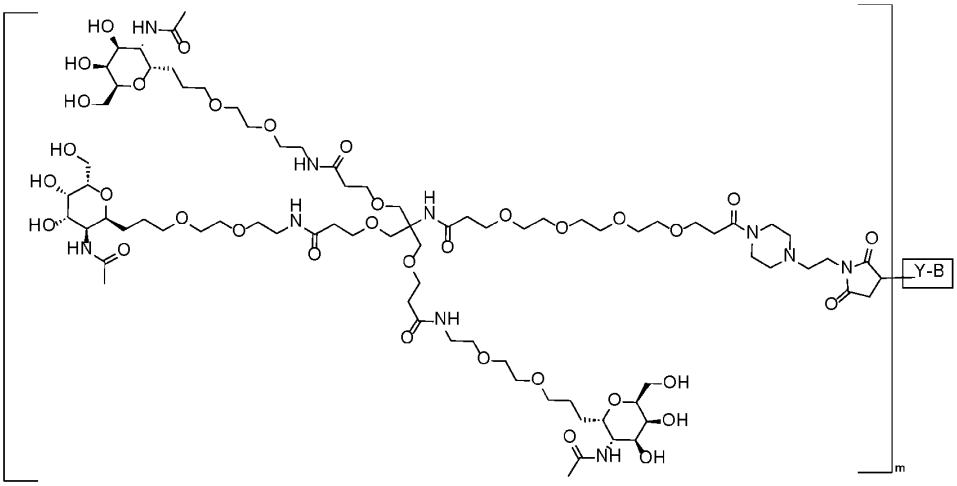
m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



wherein:

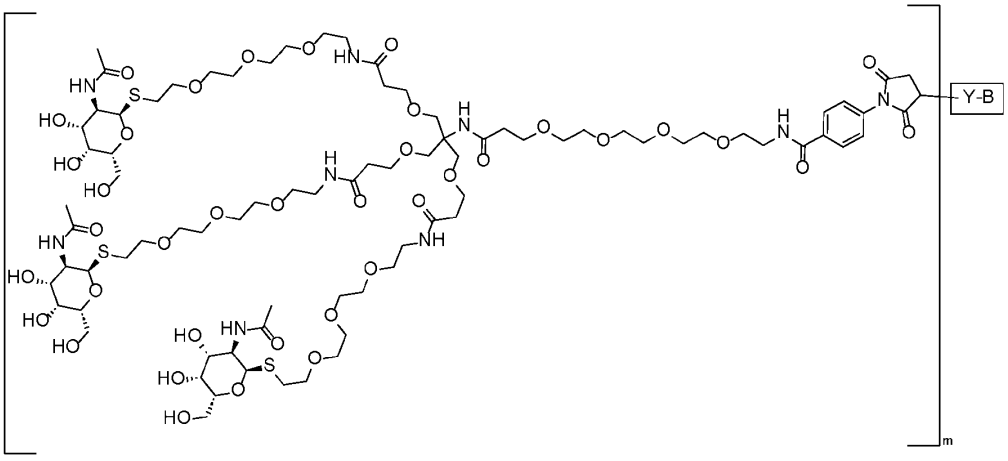
m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



wherein:

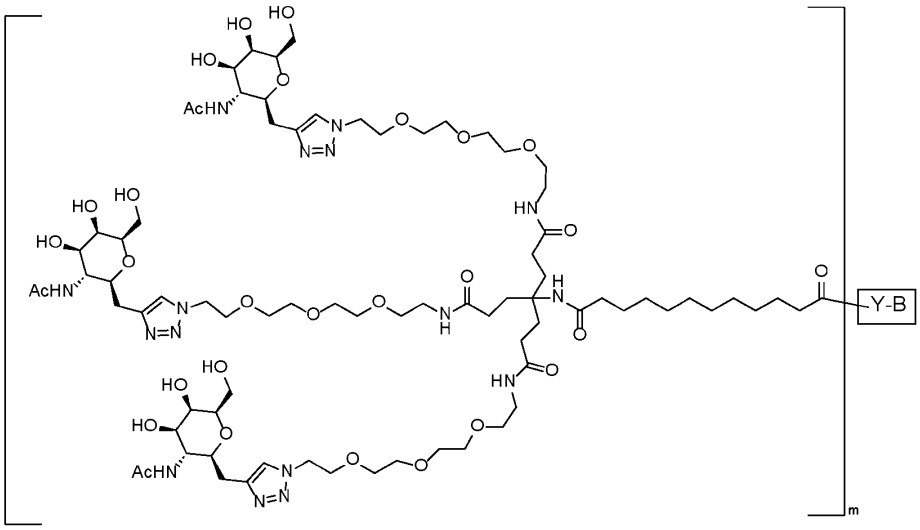
m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

In some embodiments, provided is a conjugate (i.e., LYTAC) of Formula:



wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

1.2 Pharmaceutical Compositions

In another embodiment, provided herein are pharmaceutical compositions including one or more bifunctional molecules (e.g., conjugates) disclosed herein and a pharmaceutically acceptable carrier.

In some embodiments, the pharmaceutical compositions provided herein contain therapeutically effective amounts of one or more of the conjugates provided herein, and optionally one or more additional prophylactic or therapeutic agents, in a pharmaceutically acceptable carrier.

Pharmaceutical carriers suitable for administration of the conjugates provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration.

The conjugates described herein can be formulated as the sole pharmaceutically active ingredient in the composition or can be combined with other active ingredients.

In some embodiments, the conjugate is formulated into one or more suitable pharmaceutical preparations, such as solutions, suspensions, powders, sustained release formulations or elixirs in sterile solutions or suspensions for parenteral administration, or as transdermal patch preparation and dry powder inhalers.

In compositions provided herein, a conjugate described herein may be mixed with a suitable pharmaceutical carrier. The concentration of the conjugate in the compositions can, for example, be effective for delivery of an amount, upon administration, that treats, prevents, or ameliorates a condition or disorder described herein or a symptom thereof.

In some embodiments, the pharmaceutical compositions provided herein are formulated for single dosage administration. To formulate a composition, the weight fraction of conjugate is dissolved, suspended, dispersed or otherwise mixed in a selected carrier at an effective concentration such that the treated condition is relieved, prevented, or one or more symptoms are ameliorated.

Concentrations of the conjugate in a pharmaceutical composition provided herein will depend on, *e.g.*, the physicochemical characteristics of the conjugate, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

Pharmaceutical compositions described herein are provided for administration to a subject, for example, humans or animals (*e.g.*, mammals) in unit dosage forms, such as sterile parenteral (*e.g.*, intravenous) solutions or suspensions containing suitable quantities of the compounds or pharmaceutically acceptable derivatives thereof. Pharmaceutical compositions are also provided for administration to humans and animals in unit dosage form, including oral or nasal solutions or suspensions and oil-water emulsions containing suitable quantities of a conjugate or pharmaceutically

acceptable derivatives thereof. The conjugate is, In some embodiments, formulated and administered in unit-dosage forms or multiple-dosage forms. Unit-dose forms as used herein refers to physically discrete units suitable for human or animal (*e.g.*, mammal) subjects and packaged individually as is known in the art. Each unit-dose contains a predetermined quantity of a conjugate sufficient to produce the desired therapeutic effect, in association with the required pharmaceutical carrier, vehicle or diluent. Examples of unit-dose forms include ampoules and syringes and individually packaged capsules. Unit-dose forms can be administered in fractions or multiples thereof. A multiple-dose form is a plurality of identical unit-dosage forms packaged in a single container to be administered in segregated unit-dose form. Examples of multiple-dose forms include vials, bottles of capsules or bottles. Hence, in specific aspects, multiple dose form is a multiple of unit-doses which are not segregated in packaging.

In some embodiments, the conjugates herein are in a liquid pharmaceutical formulation. Liquid pharmaceutically administrable formulations can, for example, be prepared by dissolving, dispersing, or otherwise mixing a conjugate and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline, aqueous dextrose, glycerol, glycols, and the like, to thereby form a solution or suspension. In some embodiments, a pharmaceutical composition provided herein to be administered can also contain minor amounts of nontoxic auxiliary substances such as wetting agents, emulsifying agents, solubilizing agents, and pH buffering agents and the like.

Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see, *e.g.*, Remington: The Science and Practice of Pharmacy (2012) 22nd ed., Pharmaceutical Press, Philadelphia, PA Dosage forms or compositions containing antibody in the range of 0.005% to 100% with the balance made up from non-toxic carrier can be prepared.

Parenteral administration, in some embodiments, is characterized by injection, either subcutaneously, intramuscularly or intravenously is also contemplated herein. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. The injectables, solutions and emulsions also contain one or more excipients. Suitable excipients are, for example, water, saline, dextrose, glycerol or ethanol. Other routes of administration may include, enteric administration, intracerebral administration, nasal administration, intraarterial administration, intracardiac administration, intraosseous infusion, intrathecal administration, and intraperitoneal administration.

Preparations for parenteral administration include sterile solutions ready for injection, sterile dry soluble products, such as lyophilized powders, ready to be combined with a solvent just prior to use, including hypodermic tablets, sterile suspensions ready for injection, sterile dry insoluble products ready

to be combined with a vehicle just prior to use and sterile emulsions. The solutions can be either aqueous or nonaqueous.

If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening and solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof.

Pharmaceutically acceptable carriers used in parenteral preparations include aqueous vehicles, nonaqueous vehicles, antimicrobial agents, isotonic agents, buffers, antioxidants, local anesthetics, suspending and dispersing agents, emulsifying agents, sequestering or chelating agents and other pharmaceutically acceptable substances.

Pharmaceutical carriers also include ethyl alcohol, polyethylene glycol and propylene glycol for water miscible vehicles; and sodium hydroxide, hydrochloric acid, citric acid or lactic acid for pH adjustment.

In some embodiments, intravenous or intraarterial infusion of a sterile aqueous solution containing a conjugate described herein is an effective mode of administration. Another embodiment is a sterile aqueous or oily solution or suspension containing a conjugate described herein injected as necessary to produce the desired pharmacological effect.

In some embodiments, the pharmaceutical formulations are lyophilized powders, which can be reconstituted for administration as solutions, emulsions and other mixtures. They can also be reconstituted and formulated as solids or gels.

The lyophilized powder is prepared by dissolving a conjugate provided herein, in a suitable solvent. In some embodiments, the lyophilized powder is sterile. Suitable solvents can contain an excipient which improves the stability or other pharmacological component of the powder or reconstituted solution, prepared from the powder. Excipients that can be used include, but are not limited to, dextrose, sorbitol, fructose, corn syrup, xylitol, glycerin, glucose, sucrose or other suitable agent. A suitable solvent can also contain a buffer, such as citrate, sodium or potassium phosphate or other such buffer known to those of skill in the art at, In some embodiments, about neutral pH. Subsequent sterile filtration of the solution followed by lyophilization under standard conditions known to those of skill in the art provides an example of a formulation. In some embodiments, the resulting solution will be apportioned into vials for lyophilization. Lyophilized powder can be stored under appropriate conditions, such as at about 4 °C to room temperature.

Reconstitution of this lyophilized powder with water for injection provides a formulation for use in parenteral administration. For reconstitution, the lyophilized powder is added to sterile water or other suitable carrier.

In some embodiments, the conjugates provided herein can be formulated for local administration or topical application, such as for topical application to the skin and mucous membranes, such as in the eye, in the form of gels, creams, and lotions and for application to the eye or for intracisternal or intraspinal application. Topical administration is contemplated for transdermal delivery and also for administration to the eyes or mucosa, or for inhalation therapies. Nasal solutions of the active compound alone or in combination with other pharmaceutically acceptable excipients can also be administered.

1.3 Methods of Use

Binding of the ligand moiety of the bifunctional molecule to the ASGPR can trigger internalization and lysosomal degradation of a bound target anti-TSHR autoantibody. In some embodiments, the bifunctional molecule is a conjugate of a protein that includes the polypeptide that specifically binds anti-TSHR autoantibody and a linked ligand moiety. The bifunctional molecules of this disclosure find use in reducing levels of the extracellular target molecule anti-TSHR autoantibody in a biological system or sample. The biological system can be a human subject.

The methods of using the conjugates described herein can thus remove anti-TSHR autoantibodies from the extracellular space (the extracellular milieu) of a cell in the biological system by sequestering the target protein in the cell's lysosome and degrading the target anti-TSHR autoantibodies. Removal of a target protein may refer to reduction of the amount of, or depletion of, the target protein from the extracellular space, or the extracellular milieu. In some embodiments, the biological system or sample is a cellular sample.

The term "sample" refers to an aliquot or portion taken from a source and/or provided for analysis or processing. In some embodiments, a sample is from a biological source such as a tissue, cell or component part (e.g., a body fluid, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). In some embodiments, a sample may be or include a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, or organs. In some embodiments, a sample is or includes a medium, such as a nutrient broth or gel, which may contain cellular components, such as proteins. In some embodiments, a "primary" sample is an aliquot of the

source. In some embodiments, a primary sample is subjected to one or more processing (e.g., separation, purification, etc.) steps to prepare a sample for analysis or other use.

1.3.1 Methods of Treatment

Provided herein are methods of treating a disease or disorder caused by the target anti-TSHR autoantibody in a human subject. The present disclosure thus provides methods related to using the bifunctional molecules, conjugates and compositions of this disclosure for therapeutic treatment of Graves' disease (GD) or thyroid eye disease (TED). These conditions can be treated according to the subject methods by depletion of anti-TSHR autoantibodies (e.g., M22, K1-18, K1-70, and other TSH Receptor Binding Antibodies or TRAbs) by degradation through the lysosomal pathway.

In some embodiments, the method of treating GD or TED includes administering to a subject, e.g., a human subject in need thereof, an effective amount of a bifunctional molecule, such as a conjugate having formula (I) as described herein, or pharmaceutically acceptable salt thereof, or a pharmaceutical composition including the bifunctional molecule (e.g., as described herein).

The terms "administer", "administration", or "administering" refer to the act of injecting or otherwise physically delivering a substance (e.g., a conjugate or pharmaceutical composition provided herein) to a subject or a patient (e.g., human), such as by mucosal, topical, intradermal, parenteral, intravenous, intramuscular delivery and/or any other method of physical delivery described herein or known in the art.

In some embodiments, administration is oral. In some embodiments, administration is by subcutaneous injection. In some embodiments, administration is by intravenous infusion.

The term "treating" refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of a particular disease, disorder, and/or condition (e.g., myasthenia gravis). Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

A treatment or preventive effect is evident when there is a significant improvement, often statistically significant, in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for a given compound or composition can also be judged using

an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant modulation in a marker or symptom is observed.

The terms "effective amount" or "therapeutically effective amount" refer to an amount of a therapeutic (e.g., a conjugate or pharmaceutical composition provided herein) which is sufficient to treat, diagnose, prevent, delay the onset of, reduce and/or ameliorate the severity and/or duration of a given condition, disorder or disease (e.g., myasthenia gravis) and/or a symptom related thereto. These terms also encompass an amount necessary for the reduction, slowing, or amelioration of the advancement or progression of a given disease, reduction, slowing, or amelioration of the recurrence, development or onset of a given disease, and/or to improve or enhance the prophylactic or therapeutic effect(s) of another therapy or to serve as a bridge to another therapy. In some embodiments, "effective amount" as used herein also refers to the amount of a conjugate described herein to achieve a specified result.

The term "treatment dose" refers to one or more doses of a therapeutic agent administered in the course of addressing or alleviating a therapeutic indication. Treatment doses may be adjusted to maintain a desired concentration or level of activity of a therapeutic agent in a body fluid or biological system.

In some embodiments, the treatment dose is from about 0.1 mg/kg to about 100 mg/kg. In some embodiments, the treatment dose is from about 1 mg/kg to about 10 mg/kg. In some embodiments, the treatment dose is 1 mg/kg. In some embodiments, the treatment dose is 3 mg/kg. In some embodiments, the treatment dose is 5 mg/kg. In some embodiments, the treatment dose is 10 mg/kg.

A bifunctional molecule of this disclosure and additional therapeutic agent(s) and/or therapies for GD or TED can be administered in combination. Such combinations may be in the same composition, or the additional therapeutic agents or therapies can be administered as part of a separate composition or by another method described herein.

During administration of the compounds and conjugates according to methods of this disclosure, subjects may receive standard of care (SOC) therapy for GD or TED. Standard of care therapies include, but are not limited to, antithyroid drug treatment, radioactive iodine treatment, and surgery (i.e., thyroidectomy).

The terms "subject" and "patient" are used interchangeably. A subject can be a mammal such as a non-primate (*e.g.*, cows, pigs, horses, cats, dogs, goats, rabbits, rats, mice, etc.) or a primate (*e.g.*, monkey and human), for example a human. In some embodiments, the subject is a mammal, *e.g.*, a human, diagnosed with a disease or disorder provided herein. In another embodiment, the subject is a

mammal, *e.g.*, a human, at risk of developing a disease or disorder provided herein. In some embodiments, the subject is human.

Patients treated with bifunctional molecule according to the methods of this disclosure may be screened prior to administration. The terms "patient", "subject" and "individual" are used interchangeably herein. The term "screen" refers to a review or evaluation carried out for the purpose of selection or filtration. Patients may be screened to select individuals in need of treatment. In some embodiments, subjects are screened to select individuals most likely to respond favorably to treatment.

1.4 Definitions

Unless otherwise indicated, the term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In some embodiments, the term "about" or "approximately" means within 1, 2, or 3 standard deviations. In some embodiments, the term "about" or "approximately" means within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.4%, 0.3%, 0.25%, 0.2%, 0.1% or 0.05% of a given value or range. In some embodiments, where an integer is required, the term "about" means within plus or minus 10% of a given value or range, rounded either up or down to the nearest integer.

As used herein, the phrases "for example," "for instance," "such as," or "including" are meant to introduce examples that further clarify more general subject matter. These examples are provided only as an aid for understanding the disclosure, and are not meant to be limiting in any fashion.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

The term "autoantibody" or "autoantibodies" refers to an antibody that recognizes, binds, or otherwise interacts with an antigen normally found in a subject, or a tissue or cell of a subject. Autoantibodies are abnormal antibodies which are generated by pathogenic B cells when targeting an individual's own tissue. The term "autoimmunity" refers to the presence of antibodies (which are made by B lymphocytes) and T lymphocytes directed against normal components of a person (autoantigens). These components are called autoantigens or self-antigens and typically consist of proteins (or proteins complexed to nucleic acids). The antibodies and T lymphocytes that recognize autoantigens are called "autoantibodies" and "autoreactive T cells".

The terms "protein" and "polypeptide" are used interchangeably. Proteins may include moieties other than amino acids (*e.g.*, may be glycoproteins, etc.) and/or may be otherwise processed or modified. Those of ordinary skill in the art will appreciate that a "protein" can be a complete protein chain as produced by a cell (with or without a signal sequence), or can be a protein portion thereof. Those of ordinary skill will appreciate that a protein can sometimes include more than one protein chain, for example non-covalently or covalently attached, *e.g.*, linked by one or more disulfide bonds or associated by other means. In some embodiments, a polypeptide can occur as a single chain or as two or more associated chains, e.g., may be present as a multimer, e.g., dimer, a trimer. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, by disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid, including but not limited to, unnatural amino acids, as well as other modifications known in the art. Polypeptides may contain L-amino acids, D-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, *e.g.*, terminal acetylation, amidation, methylation, etc. In some embodiments, proteins may include natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof.

An "antibody fragment" comprises a portion of an intact antibody, such as the antigen-binding or variable region of an intact antibody. Antibody fragments include, for example, Fv fragments, Fab fragments, $F(ab')_2$ fragments, Fab' fragments, scFv fragments, and VHH fragments.

An "antigen" is a moiety or molecule that contains an epitope to which an antibody can specifically bind. As such, an antigen is also specifically bound by an antibody.

An "epitope" is a term known in the art and refers to a localized region of an antigen to which an antibody can specifically bind. An epitope can be a linear epitope of contiguous amino acids or can include amino acids from two or more non-contiguous regions of the antigen.

A "pharmaceutically acceptable excipient," "pharmaceutically acceptable diluent," "pharmaceutically acceptable carrier," and "pharmaceutically acceptable adjuvant" means an excipient, diluent, carrier, and adjuvant that are useful in preparing a pharmaceutical composition that are generally safe, non-toxic and neither biologically nor otherwise undesirable, and include an excipient, diluent, carrier, and adjuvant that are acceptable for veterinary use as well as human pharmaceutical use. "A pharmaceutically acceptable excipient, diluent, carrier and adjuvant" as used in the specification and claims includes both one and more than one such excipient, diluent, carrier, and adjuvant.

A "pharmaceutical composition" is meant to encompass a composition suitable for administration to a subject, such as a mammal, especially a human. In general, a "pharmaceutical composition" is sterile, and typically free of contaminants that are capable of eliciting an undesirable response within the subject (e.g., the compound(s) in the pharmaceutical composition is pharmaceutical grade). Pharmaceutical compositions can be designed for administration to subjects or patients in need thereof via a number of different routes of administration including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, intracheal, intramuscular, subcutaneous, and the like.

The term "pharmaceutically acceptable" means being approved by a regulatory agency of the Federal or a state government, or listed in the U.S. Pharmacopeia, European Pharmacopeia or other generally recognized Pharmacopeia for use in animals, and, more particularly in humans.

The term "pharmaceutically acceptable salt" refers to those salts which are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like. Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge, et al. describes pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 66: 1-19 (1977). The salts can be prepared in situ during the final isolation and purification of the conjugate compounds, or separately by reacting the free base function or group of a compound with a suitable organic acid.

Examples of pharmaceutically acceptable salts include, but are not limited to, nontoxic acid addition salts, or salts of an amino group formed with inorganic acids.

Compounds are described using standard nomenclature. The compounds in any of the formulas described herein may be in the form of a racemate, enantiomer, mixture of enantiomers, diastereomer, mixture of diastereomers, tautomer, N-oxide, isomer; such as rotamer, as if each is specifically described unless specifically excluded by context.

As used herein, the phrase "having the formula" or "having the structure" is not intended to be limiting and is used in the same way that the term "comprising" is commonly used. The term "independently selected from" is used herein to indicate that the recited elements, e.g., R groups or the like, can be identical or different.

A dash ("-") that is not between two letters or symbols is used to indicate a point of attachment for a substituent. For example, —(C=O)NH_2 is attached through carbon of the carbonyl (C=O) group.

The present disclosure includes compounds (e.g., as described herein) with at least one desired isotopic substitution of an atom, at an amount above the natural abundance of the isotope, i.e., enriched. Isotopes are atoms having the same atomic number but different mass numbers, i.e., the same number of protons but a different number of neutrons.

Examples of isotopes that can be incorporated into compounds of the disclosure include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, chlorine and iodine such as ^{2}H, ^{3}H, ^{11}C, ^{13}C, ^{14}C, ^{15}N, ^{18}F ^{31}P, ^{32}P, ^{35}S, ^{36}Cl, and ^{125}I respectively. In one non-limiting embodiment, isotopically labelled compounds can be used in metabolic studies (with, for example ^{14}C), reaction kinetic studies (with, for example ^{2}H or ^{3}H), detection or imaging techniques, such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT) including drug or substrate tissue distribution assays, or in treatment of patients. In particular, an ^{18}F labeled compound may be particularly desirable for PET or SPECT studies. Isotopically labeled compounds of this disclosure and prodrugs thereof can generally be prepared by carrying out the procedures disclosed in the schemes or in the examples and preparations described below by substituting a readily available isotopically labeled reagent for a non-isotopically labeled reagent.

Isotopic substitutions, for example deuterium substitutions, can be partial or complete. Partial deuterium substitution means that at least one hydrogen is substituted with deuterium. In some embodiments, the isotope is 90, 95 or 99% or more enriched in an isotope at any location of interest. In one non-limiting embodiment, deuterium is 90, 95 or 99% enriched at a desired location.

In some embodiments, the substitution of a hydrogen atom for a deuterium atom can be provided in any compound of Formulas described herein. In one non-limiting embodiment, the substitution of a hydrogen atom for a deuterium atom occurs within one or more groups selected from any of R^1, R^2, R^3, R^4, R^6, R^{11}, R^{21}, R^{22}, R^{23}, R^{24}, R^{25} R, R', and R'' etc. For example, when any of the groups are, or contain for example through substitution, methyl, ethyl, or methoxy, the alkyl residue may be deuterated (in non-limiting embodiments, CDH_2, CD_2H, CD_3, CH_2CD_3, CD_2CD_3, $CHDCH_2D$, CH_2CD_3, $CHDCHD_2$, $OCDH_2$, OCD_2H, or OCD_3 etc.). In certain other embodiments, when two substituents are combined to form a cycle the unsubstituted carbons may be deuterated.

"Aliphatic" refers to a saturated or unsaturated, straight, branched, or cyclic hydrocarbon. "Aliphatic" is intended herein to include, but is not limited to, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, and cycloalkynyl moieties, and thus incorporates each of these definitions. In one embodiment, "aliphatic" is used to indicate those aliphatic groups having 1-20 carbon atoms. The aliphatic chain can be, for example, mono-unsaturated, di-unsaturated, tri-unsaturated, or polyunsaturated, or alkynyl. Unsaturated aliphatic groups can be in a cis or trans configuration. In one embodiment, the aliphatic group contains from 1 to about 12 carbon atoms, more generally from 1 to about 6 carbon atoms or from 1 to about 4 carbon atoms. In one embodiment, the aliphatic group contains from 1 to about 8 carbon atoms. In some embodiments, the aliphatic group is C_1-C_2, C_1-C_3, C_1-C_4, C_1-C_5 or C_1-C_6. The specified ranges as used herein indicate an aliphatic group having each member of the range described as

an independent species. For example, the term C_1-C_6 aliphatic as used herein indicates a straight or branched alkyl, alkenyl, or alkynyl group having from 1, 2, 3, 4, 5, or 6 carbon atoms and is intended to mean that each of these is described as an independent species. For example, the term C_1-C_4 aliphatic as used herein indicates a straight or branched alkyl, alkenyl, or alkynyl group having from 1, 2, 3, or 4 carbon atoms and is intended to mean that each of these is described as an independent species. In one embodiment, the aliphatic group is substituted with one or more functional groups that results in the formation of a stable moiety.

"Alkyl" is a branched or straight chain saturated aliphatic hydrocarbon group. In one non-limiting embodiment, the alkyl group contains from 1 to about 12 carbon atoms, more generally from 1 to about 6 carbon atoms or from 1 to about 4 carbon atoms. In one non-limiting embodiment, the alkyl contains from 1 to about 8 carbon atoms. In some embodiments, the alkyl is C_1-C_2, C_1-C_3, C_1-C_4, C_1-C_5, or C_1-C_6. The specified ranges as used herein indicate an alkyl group having each member of the range described as an independent species. For example, the term C_1-C_6 alkyl as used herein indicates a straight or branched alkyl group having from 1, 2, 3, 4, 5, or 6 carbon atoms and is intended to mean that each of these is described as an independent species and therefore each subset is considered separately disclosed. For example, the term C_1-C_4 alkyl as used herein indicates a straight or branched alkyl group having from 1, 2, 3, or 4 carbon atoms and is intended to mean that each of these is described as an independent species. Examples of alkyl include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, t-butyl, n-pentyl, isopentyl, tert-pentyl, neopentyl, n-hexyl, 2-methylpentane, 3-methylpentane, 2,2-dimethylbutane, and 2,3-dimethylbutane. In an alternative embodiment, the alkyl group is optionally substituted. The term "alkyl" also encompasses cycloalkyl or carbocyclic groups. For example, when a term is used that includes "alk" then "cycloalkyl" or "carbocyclic" can be considered part of the definition, unless unambiguously excluded by the context. For example, and without limitation, the terms alkyl, alkoxy, haloalkyl, etc. can all be considered to include the cyclic forms of alkyl, unless unambiguously excluded by context.

"Alkenyl" is a linear or branched aliphatic hydrocarbon groups having one or more carbon-carbon double bonds that may occur at a stable point along the chain. The specified ranges as used herein indicate an alkenyl group having each member of the range described as an independent species, as described above for the alkyl moiety. Examples of alkenyl radicals include, but are not limited to ethenyl, propenyl, allyl, propenyl, butenyl and 4-methylbutenyl. The term "alkenyl" also embodies "cis" and "trans" alkenyl geometry, or alternatively, "E" and "Z" alkenyl geometry. In an alternative embodiment, the alkenyl group is optionally substituted. The term "Alkenyl" also encompasses cycloalkyl or carbocyclic groups possessing at least one point of unsaturation.

"Alkynyl" is a branched or straight chain aliphatic hydrocarbon group having one or more carbon-carbon triple bonds that may occur at any stable point along the chain. The specified ranges as used herein indicate an alkynyl group having each member of the range described as an independent species, as described above for the alkyl moiety. Examples of alkynyl include, but are not limited to, ethynyl, propynyl, 1-butynyl, 2-butynyl, 3-butynyl, 1-pentynyl, 2-pentynyl, 3-pentynyl, 4-pentynyl, 1-hexynyl, 2-hexynyl, 3-hexynyl, 4-hexynyl and 5-hexynyl. In an alternative embodiment, the alkynyl group is optionally substituted. The term "Alkynyl" also encompasses cycloalkyl or carbocyclic groups possessing at least one triple bond.

"Alkylene" is a bivalent saturated hydrocarbon. Alkylenes, for example, can be a 1, 2, 3, 4, 5, 6, 7 to 8 carbon moiety, 1 to 6 carbon moiety, or an indicated number of carbon atoms, for example C_1-C_2alkylene, C_1-C_3alkylene, C_1-C_4alkylene, C_1-C_6alkylene, or C_1-C_6alkylene.

"Alkenylene" is a bivalent hydrocarbon having at least one carbon-carbon double bond. Alkenylenes, for example, can be a 2 to 8 carbon moiety, 2 to 6 carbon moiety, or an indicated number of carbon atoms, for example C_2-C_4alkenylene.

"Alkynylene" is a bivalent hydrocarbon having at least one carbon-carbon triple bond. Alkynylenes, for example, can be a 2 to 8 carbon moiety, 2 to 6 carbon moiety, or an indicated number of carbon atoms, for example C_2-C_4alkynylene.

The term "amino" refers to the group -NRR' wherein R and R' are independently hydrogen or nonhydrogen substituents, with nonhydrogen substituents including, for example, alkyl, aryl, alkenyl, aralkyl, and substituted and/or heteroatom-containing variants thereof.

"Chain" indicates a linear chain to which all other chains, long or short or both, may be regarded as being pendant. Where two or more chains could equally be considered to be the main chain, "chain" refers to the one which leads to the simplest representation of the molecule.

"Cycloalkyl" refers to cyclic alkyl groups of from 3 to 10 carbon atoms having single or multiple cyclic rings including fused, bridged, and spiro ring systems. Examples of suitable cycloalkyl groups include, for instance, cyclopropyl, cyclobutyl, cyclopentyl, cyclooctyl and the like. Such cycloalkyl groups include, by way of example, single ring structures such as cyclopropyl, cyclobutyl, cyclopentyl, cyclooctyl, and the like, or multiple ring structures such as adamantanyl, and the like.

"Halo" and "halogen" refers to fluorine, chlorine, bromine or iodine.

"Haloalkyl" is a branched or straight-chain alkyl groups substituted with 1 or more halo atoms described above, up to the maximum allowable number of halogen atoms. Examples of haloalkyl groups

include, but are not limited to, fluoromethyl, difluoromethyl, trifluoromethyl, chloromethyl, dichloromethyl, trichloromethyl, pentafluoroethyl, heptafluoropropyl, difluorochloromethyl, dichlorofluoromethyl, difluoroethyl, difluoropropyl, dichloroethyl and dichloropropyl. "Perhaloalkyl" means an alkyl group having all hydrogen atoms replaced with halogen atoms. Examples include but are not limited to, trifluoromethyl and pentafluoroethyl.

"Haloalkoxy" indicates a haloalkyl group as defined herein attached through an oxygen bridge (oxygen of an alcohol radical).

The term "heteroaliphatic" refers to an aliphatic moiety that contains at least one heteroatom in the chain, for example, an amine, carbonyl, carboxy, oxo, thio, phosphate, phosphonate, nitrogen, phosphorus, silicon, or boron atoms in place of a carbon atom. In one embodiment, the only heteroatom is nitrogen. In one embodiment, the only heteroatom is oxygen. In one embodiment, the only heteroatom is sulfur. "Heteroaliphatic" is intended herein to include, but is not limited to, heteroalkyl, heteroalkenyl, heteroalkynyl, heterocycloalkyl, heterocycloalkenyl, and heterocycloalkynyl moieties. In one embodiment, "heteroaliphatic" is used to indicate a heteroaliphatic group (cyclic, acyclic, substituted, unsubstituted, branched or unbranched) having 1-20 carbon atoms. In one embodiment, the heteroaliphatic group is optionally substituted in a manner that results in the formation of a stable moiety. Nonlimiting examples of heteroaliphatic moieties are polyethylene glycol, polyalkylene glycol, amide, polyamide, polylactide, polyglycolide, thioether, ether, alkyl-heterocycle-alkyl, —O-alkyl-O-alkyl, alkyl-O-haloalkyl, etc.

"Heterocycloalkyl" is an alkyl group as defined herein substituted with a heterocyclo group as defined herein.

"Arylalkyl" is an alkyl group as defined herein substituted with an aryl group as defined herein.

"Heteroarylalkyl" is an alkyl group as defined herein substituted with a heteroaryl group as defined herein.

The term "alkynyl" refers to a linear or branched hydrocarbon group of 2 to 24 carbon atoms containing at least one triple bond, such as ethynyl, n-propynyl, and the like. Generally, although again not necessarily, alkynyl groups herein may contain 2 to about 18 carbon atoms, and such groups may further contain 2 to 12 carbon atoms. The term "lower alkynyl" intends an alkynyl group of 2 to 6 carbon atoms. The term "substituted alkynyl" refers to alkynyl substituted with one or more substituent groups, and the terms "heteroatom-containing alkynyl" and "heteroalkynyl" refer to alkynyl in which at least one carbon atom is replaced with a heteroatom. If not otherwise indicated, the terms "alkynyl" and "lower

alkynyl" include linear, branched, unsubstituted, substituted, and/or heteroatom-containing alkynyl and lower alkynyl, respectively.

The term aryl" refers to a radical of a monocyclic or polycyclic (e.g., bicyclic or tricyclic) 4n+2 aromatic ring system (e.g., having 6, 10, or 14 π electrons shared in a cyclic array) having 6-14 ring carbon atoms and zero heteroatoms provided in the aromatic ring system ("C_{6-14} aryl"). In some embodiments, an aryl group has 6 ring carbon atoms ("C_6 aryl"; e.g., phenyl). In some embodiments, an aryl group has 10 ring carbon atoms ("C_{10} aryl"; e.g., naphthyl such as 1-naphthyl and 2-naphthyl). In some embodiments, an aryl group has 14 ring carbon atoms ("C_{14} aryl"; e.g., anthracyl). "Aryl" also includes ring systems wherein the aryl ring, as defined above, is fused with one or more carbocyclyl or heterocyclyl groups wherein the radical or point of attachment is on the aryl ring, and in such instances, the number of carbon atoms continue to designate the number of carbon atoms in the aryl ring system. The one or more fused carbocyclyl or heterocyclyl groups can be 4 to 7 or 5 to 7-membered saturated or partially unsaturated carbocyclyl or heterocyclyl groups that optionally contain 1, 2, or 3 heteroatoms independently selected from nitrogen, oxygen, phosphorus, sulfur, silicon and boron, to form, for example, a 3,4-methylenedioxyphenyl group. In one non-limiting embodiment, aryl groups are pendant. An example of a pendant ring is a phenyl group substituted with a phenyl group. In an alternative embodiment, the aryl group is optionally substituted as described above. In some embodiments, the aryl group is an unsubstituted C_{6-14} aryl. In some embodiments, the aryl group is a substituted C_{6-14} aryl. An aryl group may be optionally substituted with one or more functional groups that include but are not limited to, halo, hydroxy, nitro, amino, cyano, haloalkyl, aryl, heteroaryl, and heterocyclo.

The term "heterocyclyl" (or "heterocyclo") includes saturated, and partially saturated heteroatom-containing ring radicals, where the heteroatoms may be selected from nitrogen, sulfur and oxygen. Heterocyclic rings comprise monocyclic 3-8 membered rings, as well as 5-16 membered bicyclic ring systems (which can include bridged fused and spiro-fused bicyclic ring systems). It does not include rings containing —O—O—.—O—S— or —S—S— portions. Said "heterocyclyl" group may be optionally substituted, for example, with 1, 2, 3, 4 or more substituents that include but are not limited to, hydroxyl, Boc, halo, haloalkyl, cyano, alkyl, aralkyl, oxo, alkoxy, and amino. Examples of saturated heterocyclo groups include saturated 3- to 6-membered heteromonocyclic groups containing 1 to 4 nitrogen atoms [e.g. pyrrolidinyl, imidazolidinyl, piperidinyl, pyrrolinyl, piperazinyl]; saturated 3 to 6-membered heteromonocyclic group containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms [e.g. morpholinyl]; saturated 3 to 6-membered heteromonocyclic group containing 1 to 2 sulfur atoms and 1 to 3 nitrogen atoms [e.g., thiazolidinyl]. Examples of partially saturated heterocyclyl radicals include but are not limited to, dihydrothienyl, dihydropyranyl, dihydrofuryl, and dihydrothiazolyl. Examples of partially

saturated and saturated heterocyclo groups include but are not limited to, pyrrolidinyl, imidazolidinyl, piperidinyl, pyrrolinyl, pyrazolidinyl, piperazinyl, morpholinyl, tetrahydropyranyl, thiazolidinyl, dihydrothienyl, 2,3-dihydro-benzo[1,4]dioxanyl, indolinyl, isoindolinyl, dihydrobenzothienyl, dihydrobenzofuryl, isochromanyl, chromanyl, 1,2-dihydroquinolyl, 1,2,3,4-tetrahydro-isoquinolyl, 1,2,3,4-tetrahydro-quinolyl, 2,3,4,4a,9,9a-hexahydro-1H-3-aza-fluorenyl, 5,6,7-trihydro-1,2,4-triazolo[3,4-a]isoquinolyl, 3,4-dihydro-2H-benzo[1,4]oxazinyl, benzo[1,4]dioxanyl, 2,3-dihydro-1H-1λ′-benzo[d]isothiazol-6-yl, dihydropyranyl, dihydrofuryl and dihydrothiazolyl.

Heterocyclo groups also include radicals where heterocyclic radicals are fused/condensed with aryl or heteroaryl radicals: such as unsaturated condensed heterocyclic group containing 1 to 5 nitrogen atoms, for example, indoline, isoindoline, unsaturated condensed heterocyclic group containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, unsaturated condensed heterocyclic group containing 1 to 2 sulfur atoms and 1 to 3 nitrogen atoms, and saturated, partially unsaturated and unsaturated condensed heterocyclic group containing 1 to 2 oxygen or sulfur atoms.

The term "heteroaryl" denotes aryl ring systems that contain one or more heteroatoms selected from O, N and S, wherein the ring nitrogen and sulfur atom(s) are optionally oxidized, and nitrogen atom(s) are optionally quarternized. Examples include but are not limited to, unsaturated 5 to 6 membered heteromonocyclyl groups containing 1 to 4 nitrogen atoms, such as pyrrolyl, imidazolyl, pyrazolyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, pyrimidyl, pyrazinyl, pyridazinyl, triazolyl [e.g., 4H-1,2,4-triazolyl, IH-1,2,3-triazolyl, 2H-1,2,3-triazolyl]; unsaturated 5- to 6-membered heteromonocyclic groups containing an oxygen atom, for example, pyranyl, 2-furyl, 3-furyl, etc.; unsaturated 5 to 6-membered heteromonocyclic groups containing a sulfur atom, for example, 2-thienyl, 3-thienyl, etc.; unsaturated 5- to 6-membered heteromonocyclic groups containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, for example, oxazolyl, isoxazolyl, oxadiazolyl [e.g., 1,2,4-oxadiazolyl, 1,3,4-oxadiazolyl, 1,2,5-oxadiazolyl]; unsaturated 5 to 6-membered heteromonocyclic groups containing 1 to 2 sulfur atoms and 1 to 3 nitrogen atoms, for example, thiazolyl, thiadiazolyl [e.g., 1,2,4-thiadiazolyl, 1,3,4-thiadiazolyl, 1,2,5-thiadiazolyl].

As used herein, the terms "may," "optional," "optionally," or "may optionally" mean that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not. For example, the phrase "optionally substituted" means that a non-hydrogen substituent may or may not be present on a given atom, and, thus, the description includes structures wherein a non-hydrogen substituent is present and structures wherein a non-hydrogen substituent is not present.

The term "optionally substituted" denotes the substitution of a group herein by a moiety including, but not limited to, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_3-C_{12} cycloalkyl, C_3-

C_{12} cycloalkenyl, C_1-C_{12} heterocycloalkyl, C_3-C_{12} heterocycloalkenyl, C_1-C_{10} alkoxy, aryl, aryloxy, heteroaryl, heteroaryloxy, amino, C_1-C_{10} alkylamino, C_1-C_{10} dialkylamino, arylamino, diarylamino, C_1-C_{10} alkylsulfonamino, arylsulfonamino, C_1-C_{10} alkylimino, arylimino, C_1-C_{10} alkylsulfonimino, arylsulfonimino, hydroxyl, halo, thio, C_1-C_{10} alkylthio, 113midazoli, C_1-C_{10} alkylsulfonyl, arylsulfonyl, acylamino, aminoacyl, aminothioacyl, amidino, guanidine, ureido, cyano, nitro, azido, acyl, thioacyl, acyloxy, carboxyl, and carboxylic ester.

In one alternative embodiment any suitable group may be present on a "substituted" or "optionally substituted" position if indicated that forms a stable molecule and meets the desired purpose of the disclosure and includes, but is not limited to, e.g., halogen (which can independently be F, Cl, Br or I); cyano; hydroxyl; nitro; azido; alkanoyl (such as a C_2-C_6 alkanoyl group); carboxamide; alkyl, cycloalkyl, alkenyl, alkynyl, alkoxy, aryloxy such as phenoxy; thioalkyl including those having one or more thioether linkages; alkylsulfinyl; alkylsulfonyl groups including those having one or more sulfonyl linkages; aminoalkyl groups including groups having more than one N atoms; aryl (e.g., phenyl, biphenyl, naphthyl, or the like, each ring either substituted or unsubstituted); arylalkyl having for example, 1 to 3 separate or fused rings and from 6 to about 14 or 18 ring carbon atoms, with benzyl being an exemplary arylalkyl group; arylalkoxy, for example, having 1 to 3 separate or fused rings with benzyloxy being an exemplary arylalkoxy group; or a saturated or partially unsaturated heterocycle having 1 to 3 separate or fused rings with one or more N, O or S atoms, or a heteroaryl having 1 to 3 separate or fused rings with one or more N, O or S atoms, e.g. coumarinyl, quinolinyl, isoquinolinyl, quinazolinyl, pyridyl, pyrazinyl, pyrimidinyl, furanyl, pyrrolyl, thienyl, thiazolyl, triazinyl, oxazolyl, isoxazolyl, imidazolyl, indolyl, benzofuranyl, benzothiazolyl, tetrahydrofuranyl, tetrahydropyranyl, piperidinyl, morpholinyl, piperazinyl, and pyrrolidinyl. Such groups may be further substituted, e.g. with hydroxy, alkyl, alkoxy, halogen and amino. In some embodiments "optionally substituted" includes one or more substituents independently selected from halogen, hydroxyl, amino, cyano, —CHO, —COOH, —$CONH_2$, alkyl including C_1-C_6alkyl, alkenyl including C_2-C_6alkenyl, alkynyl including C_2-C_6alkynyl, —C_1-C_6alkoxy, alkanoyl including C_2-C_6alkanoyl, C_1-C_6alkylester, (mono- and di-C_1-C_6alkylamino)C_0-C_2alkyl, haloalkyl including C_1-C_6haloalkyl, hydoxyC_1-C_6alkyl, ester, carbamate, urea, sulfonamide, —C_1-C_6alkyl(heterocyclo), C_1-C_6alkyl(heteroaryl), —C_1-C_6alkyl(C_3-C_7cycloalkyl), O—C_1-C_6alkyl(C_3-C_7cycloalkyl), $B(OH)_2$, phosphate, phosphonate and haloalkoxy including C_1-C_6haloalkoxy.

When the term "substituted" appears prior or after a list of possible substituted groups, it is intended that the term apply to every member of that group. For example, the phrase "substituted alkyl and aryl" is to be interpreted as "substituted alkyl and substituted aryl."

In addition to the disclosure herein, the term "substituted," when used to modify a specified group or radical, can also mean that one or more hydrogen atoms of the specified group or radical are each, independently of one another, replaced with the same or different substituent groups as defined herein.

In addition to the disclosure herein, in a certain embodiment, a group that is substituted has 1, 2, 3, or 4 substituents, 1, 2, or 3 substituents, 1 or 2 substituents, or 1 substituent.

Unless indicated otherwise, the nomenclature of substituents that are not explicitly defined herein are arrived at by naming the terminal portion of the functionality followed by the adjacent functionality toward the point of attachment. For example, the substituent "hydroxyalkyl" refers to the group HO-(alkyl)-.

As to any of the groups disclosed herein which contain one or more substituents, it is understood, of course, that such groups do not contain any substitution or substitution patterns which are sterically impractical and/or synthetically non-feasible. In addition, the subject compounds include all stereochemical isomers arising from the substitution of these compounds.

In some embodiments, a substituent may contribute to optical isomerism and/or stereo isomerism of a compound.

A compound of this disclosure may form a solvate with a solvent (including water). Therefore, in one non-limiting embodiment, the present disclosure includes a solvated form of the compound. The term "solvate" refers to a molecular complex of a compound (including a salt thereof) with one or more solvent molecules. Non-limiting examples of solvents are water, ethanol, isopropanol, dimethyl sulfoxide, acetone and other common organic solvents. The term "hydrate" refers to a molecular complex comprising a compound and water. Pharmaceutically acceptable solvates in accordance with the disclosure include those wherein the solvent may be isotopically substituted, e.g. D_2O, d_6-acetone, d_6-DMSO. A solvate can be in a liquid or solid form.

Salts, solvates, hydrates, and prodrug forms of a compound are of interest. All such forms are embraced by the present disclosure. Thus, the compounds described herein include salts, solvates, hydrates, prodrug and isomer forms thereof, including the pharmaceutically acceptable salts, solvates, hydrates, prodrugs and isomers thereof. In some embodiments, a compound may be a metabolized into a pharmaceutically active derivative.

Unless otherwise specified, reference to an atom is meant to include isotopes of that atom. For example, reference to H is meant to include ^{1}H, ^{2}H (i.e., D) and ^{3}H (i.e., T), and reference to C is meant to include ^{12}C and all isotopes of carbon (such as ^{13}C).

Definitions of other terms and concepts appear throughout the detailed description.

2. EXAMPLES

The examples in this section are offered by way of illustration, and not by way of limitation.

2.1 Preparation of ASGPR ligand-linker compounds

ASGPR binding compounds and conjugates are described in International Application No. PCT/US2022/037227, filed July 14, 2022, and the disclosure of which is herein incorporated by reference in its entirety. The following are additional illustrative schemes and examples of how the compounds described herein can be prepared and tested. Although the examples can represent only some embodiments, it should be understood that the following examples are illustrative and not limiting. All substituents, unless otherwise specified, are as previously defined. The reagents and starting materials are readily available to one of ordinary skill in the art. The specific synthetic steps for each of the routes described may be combined in different ways, or in conjunction with steps from different schemes, to prepare the compounds described herein.

Example 1: Preparation of Monovalent Amine Intermediates

The following section provides details for the preparation of monovalent amine intermediate compounds which can be used in the preparation of ASGPR ligand-linker example compounds. Table 7 illustrates various monovalent amines useful for the synthesis and binding experiments.

Table 7: Monovalent Amine Intermediates for synthesis and binding experiments

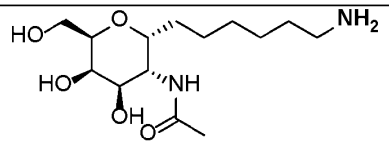
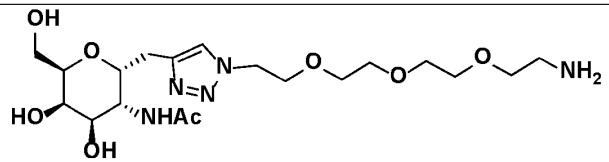
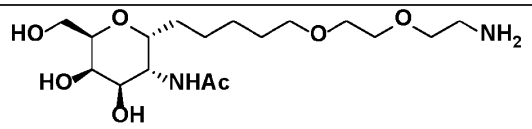
#	Structure	LCMS m/z
XB45		305.2 [M+H]+
XB46		1941.5 [M+H]+
XB47		379.2 [M+H]+

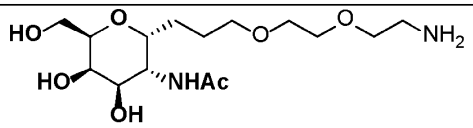
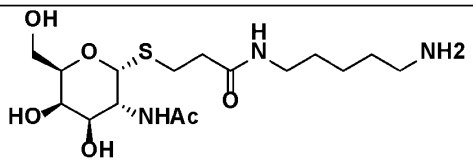
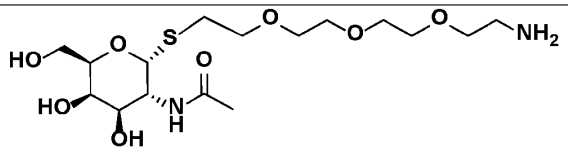
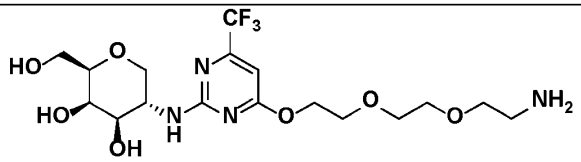
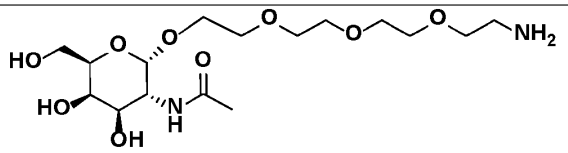
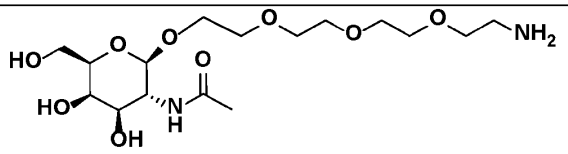
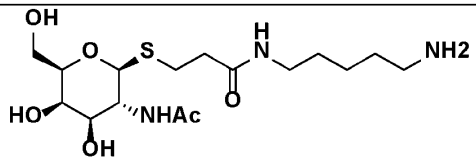
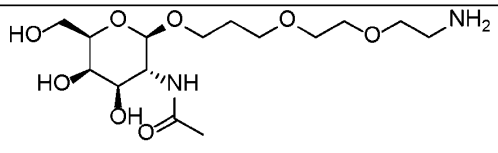
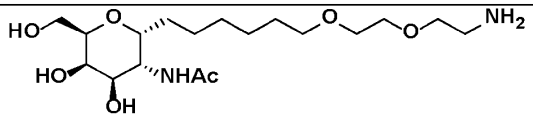
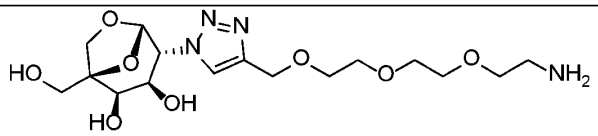
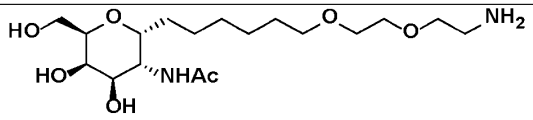
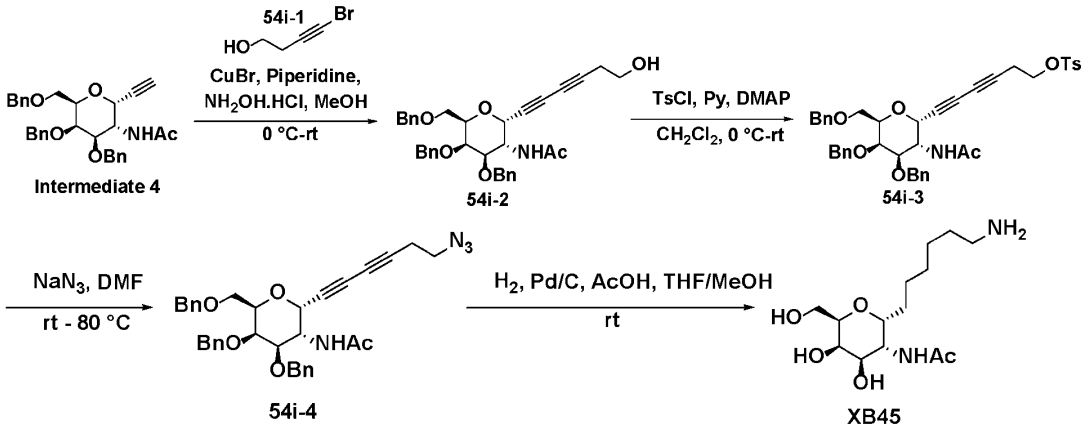
Table 7: Monovalent Amine Intermediates for synthesis and binding experiments		
#	Structure	LCMS m/z
XB48		351.2 [M+H]+
XB49		394.2 [M+H]+
XB50		413.1 [M+H]+
XB51		457.2 [M+H]+
XB52		397.2 [M+H]+
XB53		397.2 [M+H]+
XB54		394.2 $[M+H]^+$.
XB55		367.1 [M+H]+

Table 7: Monovalent Amine Intermediates for synthesis and binding experiments		
#	Structure	LCMS m/z
XB56		379.4 $[M + H]^+$
XB57		405.2 $[M + H]^+$
XB58		393.2 [M+H]+

The compounds depicted in Table 7 were prepared according to the procedures outlined herein using the appropriate starting material.

(i) Synthesis of N-((2R,3R,4R,5R,6R)-2-(6-aminohexyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB45)



To a stirred solution of N-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-ethynyltetrahydro-2H-pyran-3-yl)acetamide (**intermediate 4**, 2.0 g, 1.0 eq., 4.0 mmol), piperidine (0.852 g, 2.5 eq., 10.0 mmol), copper(I) bromide (0.056 g, 0.1 eq., 0.4 mmol), and hydroxylamine hydrochloride (0.055 g, 0.2 eq., 0.4 mmol) in methanol (8.0 mL) under nitrogen atmosphere at room temperature, was added a degassed solution of 4-bromobut-3-yn-1-ol (**54i-1**, 0.656 g, 1.1 eq., 2.2 mmol) in methanol (2

mL) over a period of 1.5 hrs. The mixture was stirred for 2 h. After completion (monitored by LCMS & TLC), solvent was removed under reduced pressure to get crude residue which was dissolved again in ethyl acetate and washed with ice cold water. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to give crude product which was purified by silica gel flash column chromatography using 0-50% ethyl acetate in hexane to afford N-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-(6-hydroxyhexa-1,3-diyn-1-yl)tetrahydro-2H-pyran-3-yl)acetamide (**54i-2**) as an off white solid. Yield: 1.9 g, 83.0%. LCMS m/z 568.13 $[M+H]^+$.

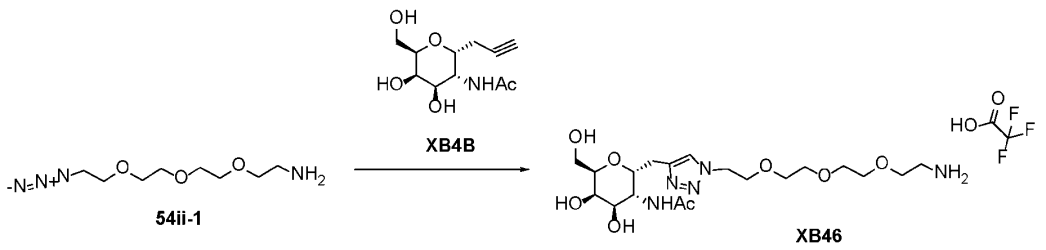
To a stirred solution of N-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-(6-hydroxyhexa-1,3-diyn-1-yl)tetrahydro-2H-pyran-3-yl)acetamide (**54i-2,** 0.80 g, 1.0 eq., 1.41 mmol) in dichloromethane (10 mL), pyridine (0.341 mL, 3.0 eq., 4.23 mmol), 4-dimethylaminopyridine (0.08 g, 0.3 eq., 0.50 mmol) and 4-methylbenzene-1-sulfonyl chloride (0.80 g, 3.0 eq., 4.23 mmol) were added sequentially at 0 °C. Then reaction mixture was stirred for 16h at room temperature. After completion (monitored by TLC), the reaction mixture was poured into cold 1N HCl, and extracted with dichloromethane. The organic part was then washed with saturated bicarbonate followed by brine, and dried over anhydrous sodium sulfate, filtered, and concentrated to give crude residue which was purified by silica gel flash column chromatography using 0-30% ethyl acetate in hexane to afford 6-((2R,3S,4R,5R,6R)-3-acetamido-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)hexa-3,5-diyn-1-yl 4-methylbenzenesulfonate (**54i-3)** as white solid. Yield: 0.81 g, 80.0%; LCMS, m/z 722.06 $[M+H]^+$.

A solution of 6-((2R,3S,4R,5R,6R)-3-acetamido-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)hexa-3,5-diyn-1-yl 4-methylbenzenesulfonate (**54i-3,** 0.715 g, 1 eq., 0.990 mmol) in N, N-dimethylformamide (5 mL) was treated with sodium azide (0.322 mg, 5 eq., 4.95 mmol). The suspension was then heated at 80°C for 6h. After completion (monitored by TLC), the reaction mixture was allowed to come to room temperature, and poured into water, and extracted with ethyl acetate. The organic part was dried over anhydrous sodium sulfate, filtered, and concentrated to give crude which was purified by silica gel flash column chromatography using 0-30% ethyl acetate in hexane to afford N-((2R,3S,4R,5R,6R)-2-(6-azidohexa-1,3-diyn-1-yl)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)acetamide (**54i-4)** as an off white solid. Yield: 0.330 g, 56.0%; LCMS m/z 593.20 $[M+H]^+$.

To a stirred solution of N-((2R,3S,4R,5R,6R)-2-(6-azidohexa-1,3-diyn-1-yl)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)acetamide (**54i-4,** 1.0 eq.,0.2 g, 0.337 mmol) in a mixture of methanol (5 mL), tetrahydrofuran (2 mL), acetic acid (0.20 mL) and water (0.20 mL) was added 10% palladium on carbon (0.30 g) and the reaction mixture was stirred at room temperature under

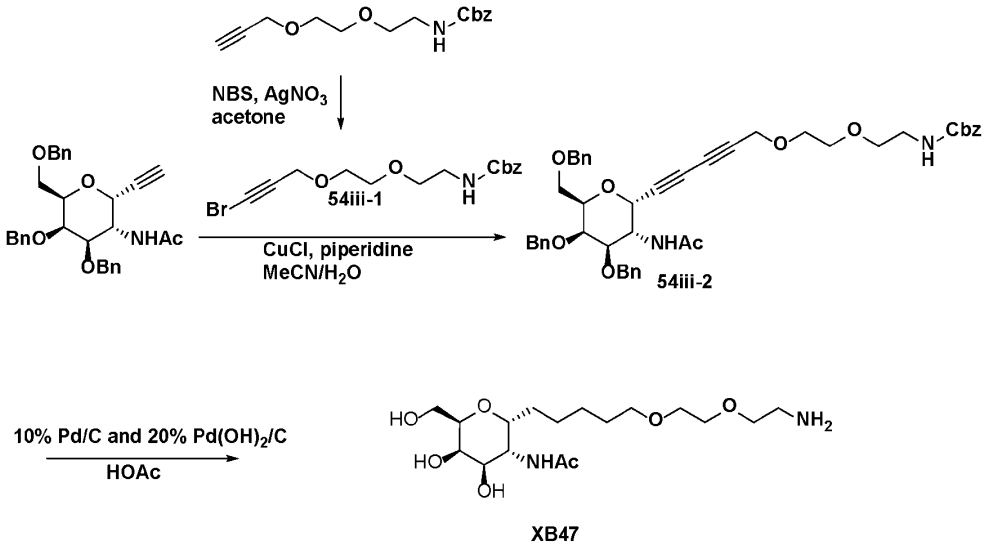
H_2 gas balloon pressure. After completion of reaction, the reaction mixture was filtered through celite bed and rinsed with methanol. The filtrate was concentrated under vacuum and purified by prep HPLC (30% acetonitrile in water with 0.1 % TFA) to afford N-((2R,3R,4R,5R,6R)-2-(6-aminohexyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide **(XB45)** Yield: 0.051 g, 49.6%; LCMS m/z 305.15 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6 with D_2O exchange) δ 4.02-3.94 (m, 1H), 3.83-3.80 (m, 1H), 3.70 (s, 1H), 3.56-3.46 (m, 4H), 2.73 (t, J = 7.6 Hz, 2H), 1.82 (s, 3H), 1.52-1.47 (m, 3H), 1.31-1.12 (m, 7H).

(ii) Synthesis of N-((2R,3R,4R,5R,6R)-2-((1-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB46)



To a solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-amine **(54ii-1)** (101.3 mg, 0.464 mmol, 1.00 eq) and N-((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-(prop-2-yn-1-yl)tetrahydro-2H-pyran-3-yl)acetamide (**XB4B)** (116.1 mg, 0.477 mmol, 1.03 eq) in 2 mL dimethyl sulfoxide was added tetrakis(acetonitrile)copper(I) hexafluorophosphate(187.3 mg, 0.403 mmol, 1.1 eq) as a solid in one portion. The mixture stirred under nitrogen atmosphere at ambient temperature for approximately 30 minutes, then directly purified by preparatory HPLC, eluting with 1-30% acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford trifluoroacetic acid salt of Compound **XB46,** as a white foam. Yield: 217 mg (81%); LCMS m/z 462.4 [M+1]+.

(iii) Synthesis of N-((2R,3R,4R,5R,6R)-2-(5-(2-(2-aminoethoxy)ethoxy)pentyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB47)



XB47

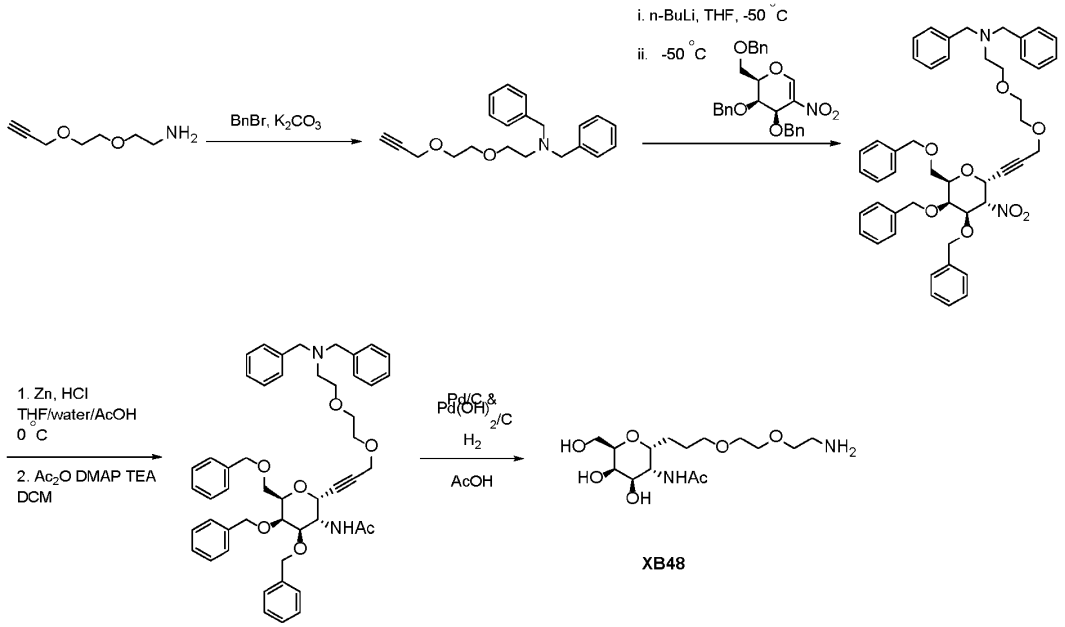
To a mixture of benzyl N-[2-(2-prop-2-ynoxyethoxy)ethyl]carbamate (1.00 eq, 816 mg, 2.94 mmol) in acetone (29 mL) were added NBS (1.34 eq, 704 mg, 3.95 mmol) and silver nitrate (0.144 eq, 72.0 mg, 0.424 mmol). The mixture was stirred at room temperature for 1h and concentrated. The residue was diluted with EtOAC and washed with water (1x). The aqueous layer was extracted with EtOAc (2x). The combined organic layers were washed with brine, dried, concentrated, purified by column (0 - 50% EtOAc/hexane) to give **54iii-1** as clear oil (930 mg, yield: 89%). LCMS m/z 378.0 $[M + Na]^+$.

To a mixture of N-[(2R,3S,4R,5R,6R)-4,5-dibenzyloxy-6-(benzyloxymethyl)-2-ethynyl-tetrahydropyran-3-yl]acetamide (1.00 eq, 201 mg, 0.403 mmol; Intermediate 4) in MeCN (7.6 mL) and water (4.4 mL) was added benzyl N-[2-[2-(3-bromoprop-2-ynoxy)ethoxy]ethyl]carbamate (**54iii-1**, 1.20 eq, 172 mg, 0.484 mmol). The mixture was cooled to 0 °C and piperidine (5.00 eq, 0.20 mL, 2.02 mmol) was added. The mixture was purged with N_2 and CuCl (0.240 eq, 9.6 mg, 0.0967 mmol) was added. The mixture was slowly warmed to room temperature and stirred at room temperature overnight. The mixture was diluted with EtOAc, washed with 10% citric acid (1x) and brine (1x), dried, concentrated, purified by column (0 -80% EtOAC/hexane) to give **54iii-2** as a white solid (232.2 mg, yield: 74%). LCMS m/z 775.2 $[M + H]^+$.

To a mixture of benzyl N-[2-[2-[5-[(2R,3S,4R,5R,6R)-3-acetamido-4,5-dibenzyloxy-6-(benzyloxymethyl)tetrahydropyran-2-yl]penta-2,4-diynoxy]ethoxy]ethyl]carbamate (**54iii-2**, 1.00 eq, 232 mg, 0.300 mmol) in HOAc (6 mL) were added10% Pd/C (220 mg) and 20% $Pd(OH)_2/C$ (230 mg). The

mixture was stirred at room temperature under hydrogen for 2.5h, filtered, concentrated, and purified by prep. HPLC (2 - 40% MeCN/20 mM NH_4OH aqueous solution) to give **XB47** as a white solid (83.6 mg, yield: 74%). LCMS m/z 379.3 $[M + H]^+$.

(iv) Synthesis of N,N-dibenzyl-2-(2-((3-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-3-nitrotetrahydro-2H-pyran-2-yl)prop-2-yn-1-yl)oxy)ethoxy)ethan-1-amine (XB48)



A mixture of 2-[2-(2-Propynyloxy)ethoxy]ethylamine (1.00 eq, 9.75 g, 68.1 mmol), anhydrous potassium carbonate (2.32 eq, 21.82 g, 158 mmol) and acetonitrile (306.46 mL) was treated with benzyl bromide (2.10 eq, 17 mL, 143 mmol) then heated to 50 °C for 2h. The reaction was filtered through celite and the filtrate concentrated under vacuum. The residue was adsorbed to silica then purified by column chromatography (5-100% EtOAc in hexanes) to give N,N-dibenzyl-2-(2-prop-2-ynoxyethoxy)ethanamine as a clear oil. Yield: 18.4 g, 83%. LCMS m/z 324.22 $[M+H]^+$.

N,N-dibenzyl-2-(2-prop-2-ynoxyethoxy)ethanamine (1.43 eq, 3.70 g, 11.4 mmol) was dissolved in 10 mL toluene then concentrated to dryness and left under high vacuum. Next, (2R,3R,4R)-3,4-dibenzyloxy-2-(benzyloxymethyl)-5-nitro-3,4-dihydro-2H-pyran (1.00 eq, 3.70 g, 8.02 mmol) was dissolved in 10 mL toluene and concentrated under high vacuum. In an oven dried flask, a solution of (114-13) N,N-dibenzyl-2-(2-prop-2-ynoxyethoxy)ethanamine (1.43 eq, 3.70 g, 11.4 mmol) in anhydrous THF (31.814 mL) under nitrogen via balloon was cooled in a -50 °C (dry-ice bath - 1:1

MeOH/water) then treated with the slow addition of butyl lithium, 2.5M in hexanes (1.20 eq, 3.8 mL, 9.62 mmol) and the reaction was stirred @ -50 °C for 60 minutes. Next, a solution of (2R,3R,4R)-3,4-dibenzyloxy-2-(benzyloxymethyl)-5-nitro-3,4-dihydro-2H-pyran (1.00 eq, 3.70 g, 8.02 mmol) in dry THF (700 uL) was added dropwise over 5 minutes while maintaining -50 °C. After 60 minutes, the reaction was quenched with the addition of 7.1M aq. Ammonium chloride (32.8 eq, 37 mL, 263 mmol) (pH 9-10) then the cold bath was removed and the slurry was warmed to room temperature. Desired product was extracted with EtOAc (100 mL, 50 mL) and the combined organic layer was dried over Na2SO4, filtered and concentrated under reduced pressure to give crude oil. The oil was adsorbed to silica gel then purified by silica gel chromatography (10% then 20% 2-MeTHF in hexanes) to give: N,N-dibenzyl-2-[2-[3-[(2R,3S,4R,5R,6R)-4,5-dibenzyloxy-6-(benzyloxymethyl)-3-nitro-tetrahydropyran-2-yl]prop-2-ynoxy]ethoxy]ethanamine (2.19 g, 2.79 mmol, 35 % yield). LCMS m/z 785.34 $[M+H]^+$.

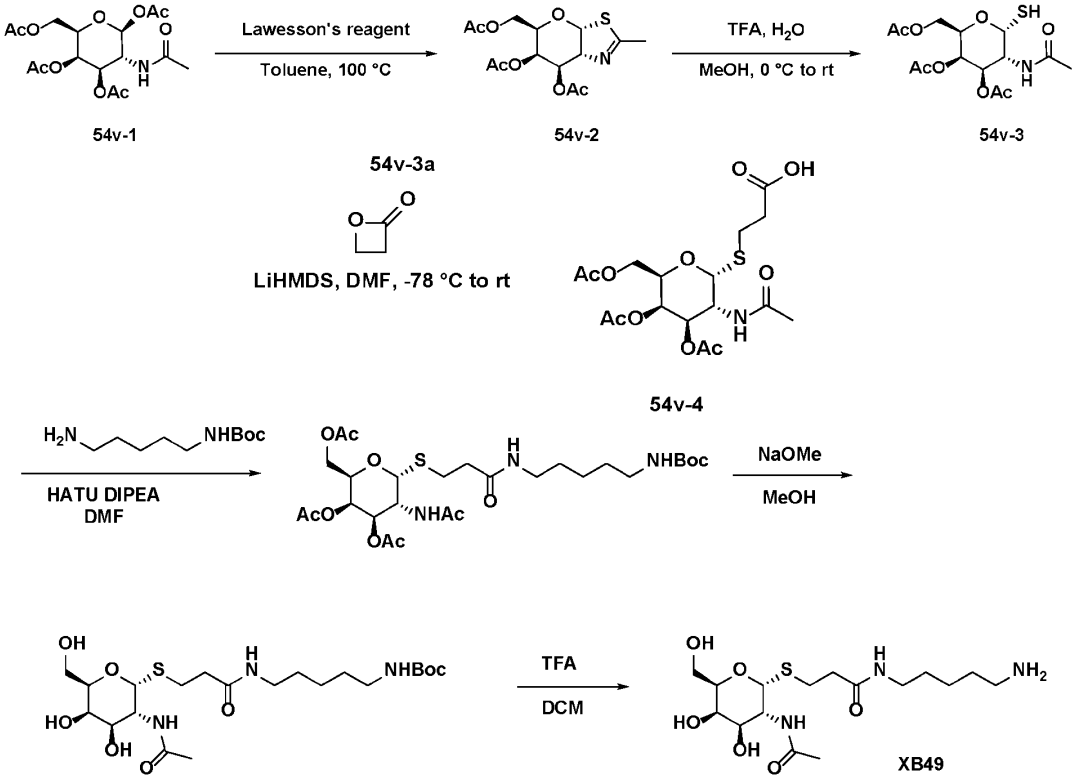
A solution of N,N-dibenzyl-2-[2-[3-[(2R,3S,4R,5R,6R)-4,5-dibenzyloxy-6-(benzyloxymethyl)-3-nitro-tetrahydropyran-2-yl]prop-2-ynoxy]ethoxy]ethanamine (1.00 eq, 2.40 g, 3.06 mmol) in THF (182.03 mL), water (77.673 mL) and acetic acid (46.055 mL) was cooled over ice then treated with zinc dust (18.8 eq, 3.76 g, 57.5 mmol) followed by 12M HCl (44.3 eq, 11 mL, 135 mmol). After 90m, the reaction was filtered then re-cooled in an ice bath before it was treated with 5M sodium hydroxide (350 eq, 214 mL, 1070 mmol) at such a rate as to keep the internal temperature below 24 °C. Next, the layers were partitioned then the aqueous layer was washed with DCM (80mL) - LCMS-Org1(initial partition), Aq2, Org2 (DCM). The aqueous layer was washed with DCM (50mL) then the combined organic layer was washed with brine then dried over Na2SO4, filtered and concentrated under reduced pressure and left under high vacuum.

The crude amine from step 3 (2.3 g) was dissolved in DCM (20 mL) before adding triethylamine (6.00 eq, 2557 uL, 18.3 mmol), 4-(Dimethylamino)pyridine (0.050 eq, 18.7 mg, 0.153 mmol) then acetic anhydride (9.80 eq, 2.8 mL, 30.0 mmol). After several hours, the reaction was quenched with water (20 mL) for several minutes. The organic layer was collected, and the aqueous layer was washed with DCM (2x10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in the presence of silica gel for purification by silica gel chromatography (0%, 5%, 10%, 15% 2MeTHF in DCM) to give N-[(2R,3S,4R,5R,6R)-4,5-dibenzyloxy-6-(benzyloxymethyl)-2-[3-[2-[2-(dibenzylamino)ethoxy]ethoxy]prop-1-ynyl]tetrahydropyran-3-yl]acetamide. Yield: 1.86 g, 76%. LCMS m/z 797.5 $[M+H]^+$.

A mixture of N-[(2R,3S,4R,5R,6R)-4,5-dibenzyloxy-6-(benzyloxymethyl)-2-[3-[2-[2-(dibenzylamino)ethoxy]ethoxy]prop-1-ynyl]tetrahydropyran-3-yl]acetamide (1.00 eq, 3.45 g, 4.33 mmol), palladium hydroxide (0.500 eq, 3.04 g, 2.16 mmol) and 10% Pd/C, Evonik Noblyst (0.500 eq,

4.61 g, 2.16 mmol) in acetic acid (86.576 mL) under nitrogen was evacuated then back-filled with hydrogen gas via balloon - a process that was repeated 3 times before leaving under an atmosphere of hydrogen. After 3 hours, the reaction was filtered over a pad of celite, the filter cake was rinsed while stirring with 100 mL methanol. Solvents were removed under reduced pressure and the residue was azeotroped with toluene then left under high vacuum. The residue was purified by RPHPLC (5-30% acetonitrile in water w/ 20mM NH4OH) and fractions lyophilized to give 927 mg of N,N-dibenzyl-2-(2-((3-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-3-nitrotetrahydro-2H-pyran-2-yl)prop-2-yn-1-yl)oxy)ethoxy)ethan-1-amine, **XB48**, as a white solid (61% yield).

(v) Synthesis of 3-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)-N-(5-aminopentyl)propenamide (XB49)



To a solution of (2S,3R,4R,5R,6R)-3-acetamido-6-(acetoxymethyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (**54v-1**, 1.0 eq, 4.0 g, 10.3 mmol) in toluene (33.2 mL), Lawesson's reagent (0.85 eq, 3.53 g, 8.73 mmol) was added and reaction mixture was heated at 100 °C for 1.5 h. After completion, reaction mixture was cooled, water was added, neutralized with solid sodium bicarbonate and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to get crude

which was purified by column chromatography using silica gel (100-200 mesh) and 0-20 % ethyl acetate in dichloromethane to afford (3aR,5R,6R,7R,7aR)-5-(acetoxymethyl)-2-methyl-3a,6,7,7a-tetrahydro-5H-pyrano[3,2-d]thiazole-6,7-diyl diacetate (**54v-2**) as a light yellow viscous liquid. Yield: 2.0 g, 56.37 %; LCMS m/z 346.10 $[M+18]^+$.

A solution of (3aR,5R,6R,7R,7aR)-5-(acetoxymethyl)-2-methyl-3a,6,7,7a-tetrahydro-5H-pyrano[3,2-d]thiazole-6,7-diyl diacetate (**54v-2**, 1.0 eq, 1.6 g, 4.63 mmol) in methanol (16 mL) and water (0.16 mL) was cooled at 0 °C, trifluoroacetic acid (0.16 mL) was added and reaction mixture was stirred at room temperature for 16 h. After completion, reaction mixture was concentrated, azeotroped with toluene (2-3 times) and dried to get afford (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-mercaptotetrahydro-2H-pyran-3,4-diyl diacetate (**54v-3**) as a light-yellow viscous liquid. Yield: 1.6 g (Crude); LCMS m/z 364.10 $[M+1]^+$.

A solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-mercaptotetrahydro-2H-pyran-3,4-diyl diacetate (**54v-3**, 1.0 eq, 2.4 g, 6.6 mmol) in N,N-dimethylformamide (24 mL) was cooled at -78 °C, Lithium bis(trimethylsilyl)amide (LiHMDS, 1M in tetrahydrofuran) (1.0 eq, 6.6 mL, 6.6 mmol) was added and reaction mixture was stirred at the same temperature for 1 h. Then, oxetan-2-one (**54v-3a**, 1.3 eq, 0.617 g, 8.58 mmol) was added and reaction mixture stirred at room temperature for 16 h. After completion, reaction mixture was concentrated to get crude which was first purified by column chromatography using silica gel (100-200 mesh) and 0-10 % methanol in dichloromethane and then by prep HPLC (10-25 % acetonitrile in water with 0.1 % trifluoroacetic acid). Fractions containing the desired product were combined and lyophilized to dryness to afford 3-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoic acid (Cpd. No. **54v-4**) as a white solid. Yield: 0.732 g, 25.68 %; LCMS m/z 436.05 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.26 (bs, 1H), 8.15 (d, J = 7.2 Hz, 1H), 5.60 (d, J = 5.2 Hz, 1H), 5.34 (d, J = 2.4 Hz, 1H), 4.88 (dd, J = 12.0 Hz & 3.2 Hz, 1H), 4.45 (t, J = 6.8 Hz, 1H), 4.40-4.34 (m, 1H), 4.09-4.00 (m, 2H), 2.76-2.64 (m, 2H), 2.54 (d, J = 7.2 Hz, 2H), 2.10 (s, 3H), 2.10 (s, 3H), 1.90 (s, 3H), 1.80 (s, 3H).

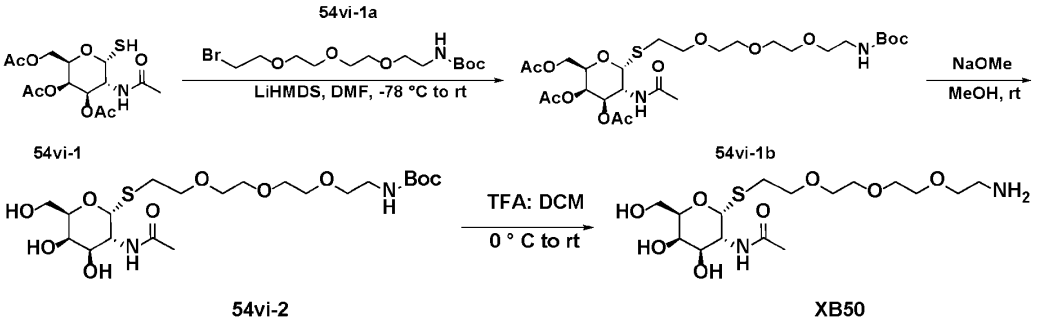
A solution of 3-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoic acid (1.00 eq, 100 mg, 0.230 mmol) and tert-butyl (5-aminopentyl)carbamate (1.1 eq, 51.1 mg, 0.253 mmol) and Diisopropylethylamine (DIPEA) (3.0 eq, 0.12 mL, 0.689 mmol) in DMF (1.15 mL) was cooled in an ice bath before adding HATU (1.2 eq, 105 mg, 0.276 mmol). After 45 minutes, the reaction was diluted with water (5 mL) and brine (5 mL) and the products were extracted with EtOAc (2x5 mL). The partitioned aqueous layer was washed with EtOAc (5 mL) and the combined organic layer was washed with citric acid then with sodium bicarbonate before

being dried over Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to give crude material that was used in the next step without further purification. LCMS m/z 620.0 $[M+H]^+$.

A solution of 25% w/w sodium methoxide in methanol (6.0 eq, 0.29 mL, 1.26 mmol) in methanol (0.839 mL) was treated with (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-((3-((5-((tert-butoxycarbonyl)amino)pentyl)amino)-3-oxopropyl)thio)tetrahydro-2H-pyran-3,4-diyl diacetate (1.0 eq, 130 mg, 0.210 mmol). After 30 minutes, the reaction was cooled in an ice bath and the reaction solution was neutralized with 12M HCl (5.9 eq, 103 uL, 1.24 mmol) then diluted with DCM (7mL) and filtered. The filtrate was concentrated under reduced pressure to give 177 mg of crude material that was used in the next step without further purification. LCMS m/z 494.0 $[M+1]^+$.

A mixture of tert-butyl (5-(3-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanamido)pentyl)carbamate (1.0 eq, 103 mg, 0.209 mmol) and trifluoroacetic acid (40.0 eq, 595 uL, 8.35 mmol) in DCM (1.04 mL) was stirred at room temp for 2 h. The reaction was concentrated under reduced pressure then dissolved in water and ammonium hydroxide for purification by reversed-phase HPLC (3-50% acetonitrile in water with 200 mM NH_4OH) to give 3-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)-N-(5-aminopentyl)propenamide, **XB49**, as a white solid. Yield: 46 mg, 56 % (over 3 steps). LCMS m/z 394.2 $[M+1]^+$.

(vi) Synthesis of N-((2R,3R,4R,5R,6R)-2-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)thio)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB50)



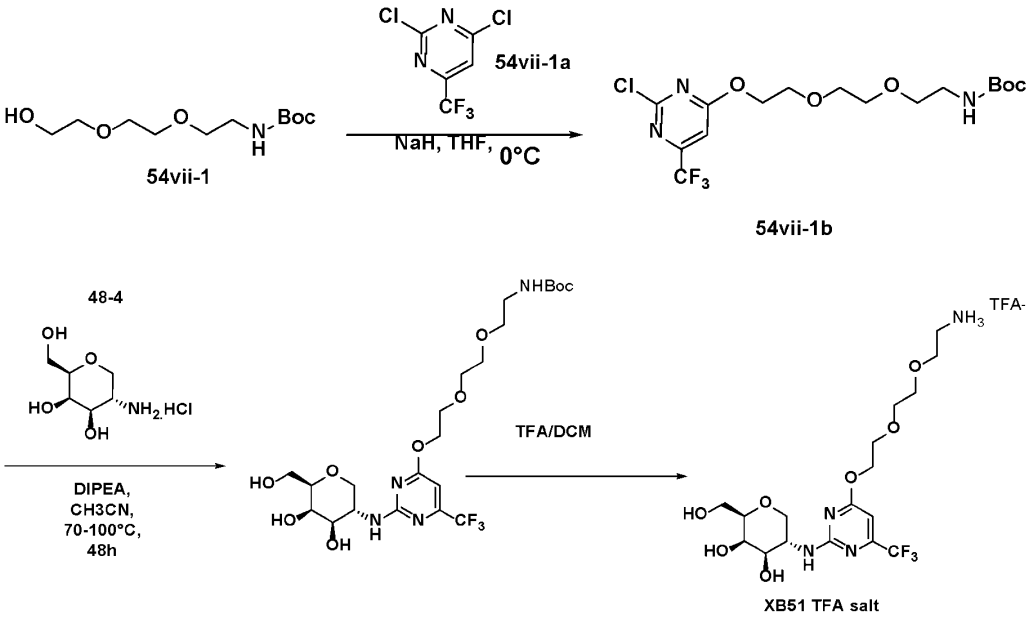
A solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-mercaptotetrahydro-2H-pyran-3,4-diyl diacetate (**54vi-1**, 1.0 eq, 0.800 g, 2.2 mmol) in N,N-dimethylformamide (8 mL) was cooled at -78 °C, Lithium bis(trimethylsilyl)amide (LiHMDS, 1M in tetrahydrofuran) (1.0 eq, 2.2 mL, 2.2 mmol) was added and reaction mixture was stirred at the same temperature for 1 h. Then, tert-butyl (2-(2-(2-bromoethoxy)ethoxy)ethoxy)ethyl)carbamate (**54vi-1a**, 1.2 eq, 0.940 g, 2.64 mmol) was added and

reaction mixture stirred at room temperature for 16 h. After completion, reaction mixture was concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-5 % methanol in dichloromethane to afford (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)thio)tetrahydro-2H-pyran-3,4-diyl diacetate (Cpd. No. **54vi-1b**) as a colourless semi solid. Yield: 0.600 g, 44.11 %; LCMS m/z 639.05 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-d_6 with D_2O) δ 5.57 (d, J = 5.2 Hz, 1H), 5.31-5.30 (m, 1H), 4.89-4.85 (m, 1H), 4.43 (t, J = 6.0 Hz, 1H), 4.36-4.32 (m, 1H), 4.06-3.97 (m, 2H), 3.60-3.56 (m, 1H), 3.54-3.51 (m, 4H), 3.36 (t, J = 6.0 Hz, 2H), 3.03 (t, J = 5.6 Hz, 2H), 2.92 (s, 2H), 2.76 (s, 1H), 2.73-2.69 (m, 1H), 2.64-2.58 (m, 1H), 2.06 (s, 3H), 1.96-1.94 (m, 5H), 1.87 (s, 3H), 1.79 (s, 3H), 1.33 (s, 9H).

A solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)thio)tetrahydro-2H-pyran-3,4-diyl diacetate (**54vi-1b**, 1.0 eq, 0.450 g, 0.705 mmol) in methanol (5 mL) was cooled at 0 °C, sodium methoxide (25 % solution in methanol) (2.0 eq, 0.33 mL, 1.41 mmol) was added and reaction mixture was stirred at room temperature for 3 h. After completion, reaction mixture was neutralized with Dowex 50WX8 hydrogen form (200-400 mesh) and filtered through sintered funnel (without celite). The filtrate was concentrated, washed with diethyl ether and dried to afford tert-butyl (2-(2-(2-(2-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)ethoxy)ethoxy)ethoxy)ethyl)carbamate (**54vi-2**) as an off white solid. Yield: 0.320 g, 88.64 %; LCMS m/z 513.10 $[M+1]^+$.

A solution of tert-butyl (2-(2-(2-(2-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)ethoxy)ethoxy)ethoxy)ethyl)carbamate (**54vi-2**, 1.0 eq, 0.320 g, 0.624 mmol) in dichloromethane (1.6 mL) was cooled at 0 °C, trifluoroacetic acid (1.6 mL) was added and reaction mixture was stirred at room temperature for 1 h. After completion, reaction mixture was concentrated, azeotroped with dichloromethane (2-3 times), washed with diethyl ether (2-3 times) and purified by prep HPLC (32-50 % acetonitrile in water with 0.1 % trifluoroacetic acid). Fractions containing the desired product were combined and lyophilized to dryness to afford N-((2R,3R,4R,5R,6R)-2-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)thio)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide, **XB50**, as a light yellow viscous liquid. Yield: 0.220 g, 85.33 %; LCMS m/z 413.10 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-*d*6) δ 7.79-7.73 (m, 3H), 5.43 (d, J = 5.6 Hz, 1H), 4.67-4.60 (m, 3H), 4.18-4.12 (m, 1H), 3.88 (t, J = 5.6 Hz, 1H), 3.73 (bs, 1H), 3.60-3.45 (m, 15H), 2.98-2.97 (m, 2H), 2.70-2.64 (m, 1H), 2.57-2.54 (m, 1H), 1.81 (s, 3H).

(vii) Synthesis of (2R,3R,4R,5S)-5-((4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-6-(trifluoromethyl)pyrimidin-2-yl)amino)-2-(hydroxymethyl)tetrahydro-2H-pyran-3,4-diol (XB51)



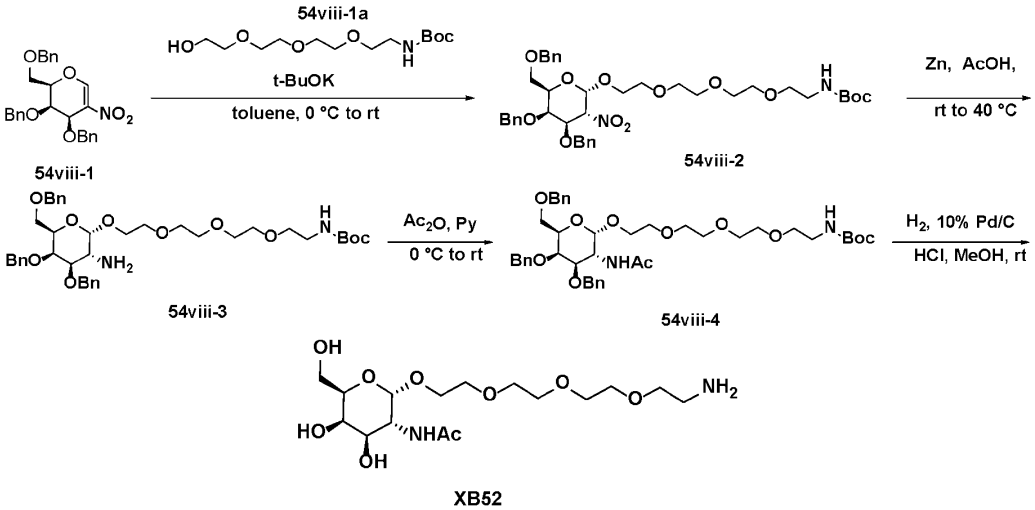
To stirred solution of *tert*-butyl (2-(2-(2-hydroxyethoxy)ethoxy)ethyl)carbamate (**54viii-1,** 1.15 g, 1.0 eq, 4.61 mmol) in tetrahydrofuran (10 mL), sodium hydride (0.277 g, 1.5 eq., 6.91 mmol, 60% in oil) was added portion wise at 0°C under N_2 and allowed to stir at 0°C for 30 minutes. Then the resultant solution was added dropwise to a solution of 2,4-dichloro-6-(trifluoromethyl)pyrimidine (1.0 g, 1.0 eq, 4.61 mmol) in tetrahydrofuran (10 mL) under N_2 atmosphere at 0°C and allowed to stir for 10 minutes. After completion (monitored by TLC), the reaction was quenched by the addition of ice, extracted with ethyl acetate, washed with brine, dried over anhydrous sodium sulfate and concentrated to get crude which was passed through silica gel (silica gel 100-200 mesh, eluent: 10% ethyl acetate in hexane) to get crude product. (1.57 g, 3.65 mmol) as light yellow liquid which was further purified by SFC to get tert-butyl (2-(2-(2-((2-chloro-6-(trifluoromethyl)pyrimidin-4-yl)oxy)ethoxy)ethoxy)ethyl)carbamate (Cpd. No. **54vii-1b**) as colourless oil. Yield: 0.60 g, 35%; LCMS m/z 430.3 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.64 (s, 1H), 6.75 (t, J = 4.8 Hz, 1H), 4.56-4.54 (m, 2H), 3.78-3.76 (m, 2H), 3.58-3.56 (m, 2H), 3.51-3.48 (m, 2H), 3.38 (t, J = 6.0 Hz, 2H), 3.06-3.02 (m, 2H), 1.35 (s, 9H).

SFC Purification Method: Column: AMYLOSE-1 (250*4.6)mm, 5um; Mobile Phase: CO2:0.1% IPAmine in HEXANE:IPA 50:50 (20:80); Flow Rate: 3.0 mL/min; Column Temperature: 40°C.

A suspension of tert-butyl N-[2-[2-[2-[2-chloro-6-(trifluoromethyl)pyrimidin-4-yl]oxyethoxy]ethoxy]ethyl]carbamate (1.00 eq, 51.0 mg, 0.119 mmol), (2R,3R,4R,5S)-5-amino-2-(hydroxymethyl)tetrahydropyran-3,4-diol;hydrochloride (**48-4**, 2.00 eq, 47.4 mg, 0.237 mmol), Diisopropylethylamine (DIPEA) (4.00 eq, 0.083 mL, 0.475 mmol), in 420 uL of anhydrous acetonitrile was heated to 75C under N_2 in a sealed vial. The mixture was heated for 1 hr, added 1 eq of DIEA (20 uL) and heated overnight at 80C, then heated at 95 °C for 24 hours. The mixture was cooled, diluted with water and formic acid, and the crude material was purified by Prep HPLC, eluting from a C18 column with a gradient of 10-100% CH3CN:water + 0.1% FA to give 51 mg of a white powder [77% yield]; LCMS: 557.1 [M+H].

A solution of tert-butyl N-[2-[2-[2-[2-[[(3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]amino]-6-(trifluoromethyl)pyrimidin-4-yl]oxyethoxy]ethoxy]ethyl]carbamate (1.00 eq, 31.0 mg, 0.0557 mmol) in 6 mL DCM was cooled to 0C and 2mL of TFA was added. The solution was stirred for 1 hr at room temperature, at which time the solution was concentrated to a crude residue which was subsequently lyophilized from water to give (2R,3R,4R,5S)-5-((4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-6-(trifluoromethyl)pyrimidin-2-yl)amino)-2-(hydroxymethyl)tetrahydro-2H-pyran-3,4-diol, **XB51 TFA salt**: 37.3 mg of a white solid (crude, TFA salt); LCMS 457.2 [M+1].

(viii) Synthesis of N-((2S,3R,4R,5R,6R)-2-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB52)



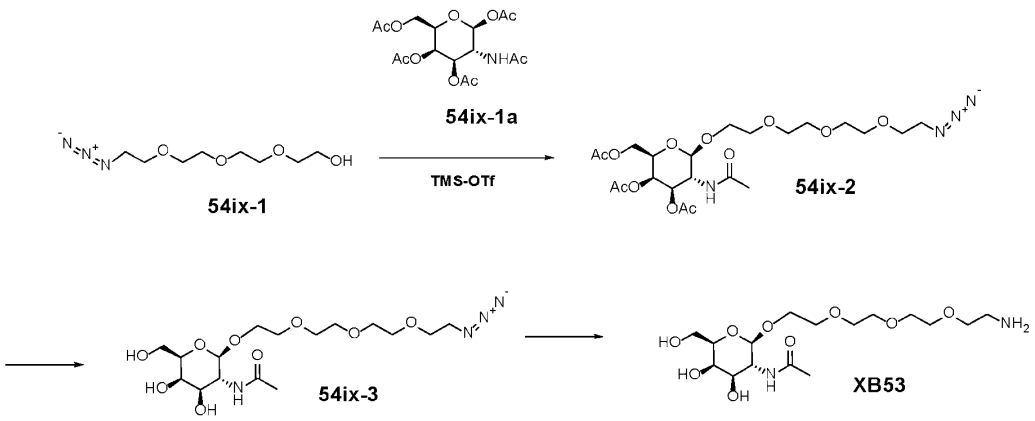
To a stirred solution of (2R,3R,4R)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran (**54viii-1**, 5.0 g, 1.0 eq., 10.8 mmol) and tert-butyl (2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl)carbamate (**54viii-1a,** 4.77 g, 1.5 eq., 16.3 mmol) in anhydrous toluene (40 mL) under Ar, activated molecular sieves (3 Å, 1.50 g) were added and the mixture stirred for 1h at room temperature Thereafter, *t*-BuOK (0.608 g, 0.5 eq., 5.42 mmol, 1M solution in THF) was added at 0°C, and stirred for 12h at room temperature. After completion (monitored by LCMS, & TLC), acetic acid (0.05 mL) was added to quench the reaction. Molecular sieves were filtered off and the filtrate was removed under reduced pressure to afford crude which was purified by silica gel flash column chromatography to (using 0-70% ethyl acetate in hexane) to afford *tert*-butyl (2-(2-(2-(2-(((2S,3R,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-3-nitrotetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethyl)carbamate (54viii-2) as colorless syrup. Yield: 2.5 g, 30.0%; LCMS m/z 755.37 $[M+H]^+$.

To a solution of *tert*-butyl (2-(2-(2-(2-(((2S,3R,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-3-nitrotetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethyl)carbamate (54viii-2, 2.5 g, 3.31 mmol, 1.0 eq.) in glacial acetic acid (30 mL); zinc (2.6 g, 12.0 eq., 39.7 mmol) was added and then heated at 40°C for 3h. After completion (monitored by TLC), reaction mixture was diluted with methanol and was filtered through *celite* pad. The volatiles were evaporated out on high vacuum to yield crude *tert*-butyl (2-(2-(2-(2-(((2S,3R,4R,5R,6R)-3-amino-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethyl)carbamate **(54viii-3)** as syrup which was used for next step without further purification. Yield: 2.5 g (crude); LCMS m/z 725.65 $[M+H]^+$.

To a solution of *tert*-butyl (2-(2-(2-(2-(((2S,3R,4R,5R,6R)-3-amino-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethyl)carbamate (**54viii-3,** 2.5 g, 3.45 mmol, 1.0 eq.) in pyridine (20 mL), acetic anhydride (10 mL) was added at 0°C and stirred the reaction mixture for 16h at room temperature. After completion, the volatiles were evaporated under reduced pressure to get crude which was purified by silica gel flash column chromatography (40-60% ethyl acetate/hexane) to afford *tert*-butyl (2-(2-(2-(2-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethyl)carbamate (Cpd. No. **54viii-4)** as off-white semi solid Yield: 2.5 g, 94.52%.; LCMS m/z 767.50 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.82 (d, J = 8.8 Hz, 1H), 7.35-7.23 (m, 15H), 6.74 (t, J = 5.6 Hz, 1H), 5.75 (s, 1H), 4.76-4.70 (m, 3H), 4.59-4.43 (m, 4H), 4.29-4.23 (m, 1H), 4.04 (bs, 1H), 3.94 (t, J = 6.4 Hz, 1H), 3.75 (dd, J = 11.2, 2.4 Hz 1H), 3.68-3.63 (m, 1H), 3.58-3.54 (m, 4H), 3.53-3.47 (m, 9H), 3.37-3.34 (m, 2H), 3.05 (q, J = 6.0 Hz, 2H), 1.83 (s, 3H), 1.36 (s, 9H).

To a solution of *tert*-butyl (2-(2-(2-(2-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethyl)carbamate **(54viii-3,** 1.0 g, 1.0 eq., 1.3 mmol) in methanol (15 mL), 10% Pd/C (1.0 g), and concentrated HCl (0.083 mL, 2.0 eq., 2.61 mmol) were added. Then reaction mixture was stirred at room temperature under H_2 gas balloon pressure for 48h. After completion, the reaction mixture was filtered on *celite* pad and washed the pad with methanol. The volatiles were evaporated in high vacuum to afford crude which was purified by prep-HPLC (25% acetonitrile in water with 0.1 % TFA) to afford N-((2S,3R,4R,5R,6R)-2-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide **(XB52*)*** as light brown semi solid Yield: 0.109 g., 21.09%; LCMS m/z 397.15 $[M+H]^+$ ^1H NMR (400 MHz, DMSO-d_6 with D_2O exchange) δ 4.70 (d, J = 2.0 Hz, 1H), 4.01-3.98 (m, 1H), 3.77-3.72 (m, 2H), 3.63-3.44 (m, 16H), 2.95 (t, J = 4.8 Hz, 2H), 1.84 (s, 3H).

(ix) Synthesis of N-((2R,3R,4R,5R,6R)-2-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB53)

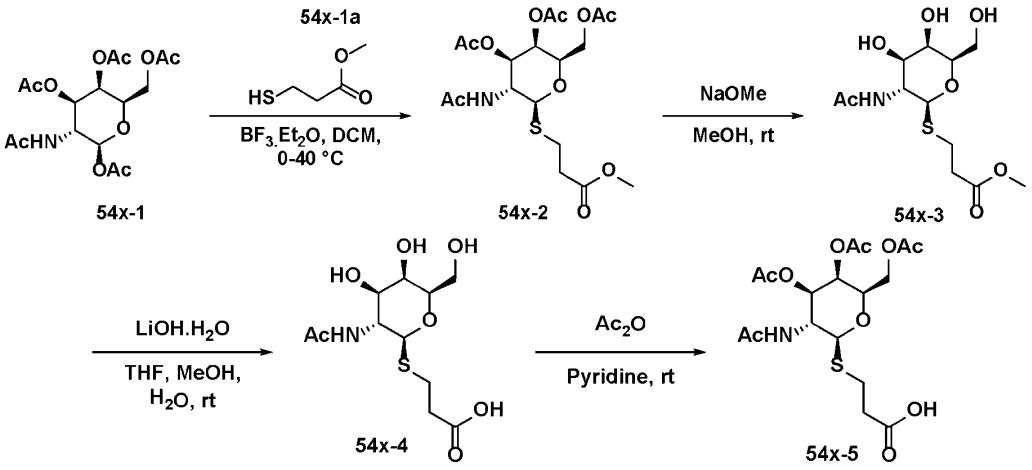


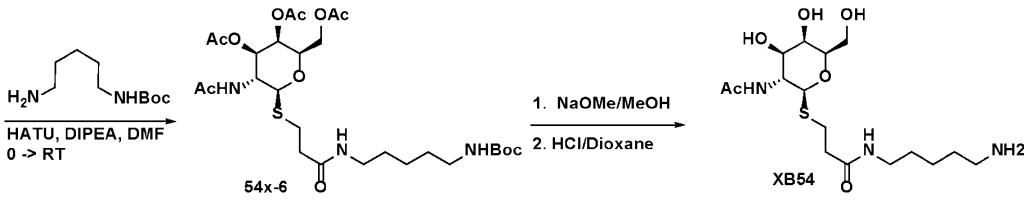
To a solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol **(54ix-1)** (246 mg, 1.12 mmol, 0.9eq.) and (2S,3R,4R,5R,6R)-3-acetamido-6-(acetoxymethyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate **(54ix-1a)** (486 mg, 1.24 mmol, 1.0 eq.) in 5 mL of 1,2-dicholomethane, stirring at ambient temperature under nitrogen atmosphere, was slowly added trimethylsilyl trifluoromethanesulfonate (45 mL, 0.25 mmol, 0.2 eq.). The mixture stirred at ambient temperature for approximately 5 minutes, then was heated to 60C. After 3 hrs., the mixture was cooled to ambient temperature and quenched with addition of triethylamine (70 mL, 0.51 mmol, 0.4 eq.). The reaction mixture was diluted further with 1,2-dicholomethane, evaporated onto silica, and purified by flash column chromatography column, eluting with 0-100% ethyl acetate/dichloromethane to afford Compound **54ix-2** as a clear thick syrup. Yield: 520 mg (75%); LCMS m/z 548.97 [M+H]+.

To a solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)tetrahydro-2H-pyran-3,4-diyl diacetate **(54ix-2)** (492 mg, 0.897 mmol) in 6 mL of methanol, stirring under nitrogen atmosphere at 0-5C, was added sodium methoxide (25% w/w in methanol) (1359 mg, 6.29 mmol, 7.0 eq) diluted in 3 mL of methanol. The reaction mixture stirred at ambient temperature for 30 minutes, at which time 8 mL of 1N aqueous hydrochloric acid was added slowly to achieve approximate final pH of 1-2. The reaction mixture was concentrated to approximately ¼ volume and purified by preparatory HPLC, eluting with 1-30% acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford Compound **54ix-3** as white solid. Yield: 244 mg (64%); LCMS m/z 423.06 [M+1]+.

To a solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethoxy)tetrahydro-2H-pyran-3,4-diyl diacetate **(54ix-3)** (239 mg, 0.566 mmol) in 15 mL methanol was added 10% w/w palladium on carbon (128 mg). The mixture was degassed under vacuum then stirred under hydrogen atmosphere via balloon. After approximately 15 minutes, the solution was filtered over Celite and washed with methanol. The filtrate was concentrated to residue, then co-evaporated from acetonitrile to afford **XB53**, as a clear oil. Yield: 243 mg (108%); LCMS m/z 397.25 [M+1]+.

(x) Synthesis of 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)-N-(5-aminopentyl)propenamide (XB54)





A solution of (2S,3R,4R,5R,6R)-3-acetamido-6-(acetoxymethyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (**54x-1**, 1.0 eq, 5.0 g, 12.8 mmol) and methyl 3-mercaptopropanoate (**54x-1a**, 2.0 eq, 3.09 mL, 25.7 mmol) in dichloromethane (50 mL) was cooled at 0 °C, boron trifluoride diethyl etherate (5.0 eq, 8.28 mL, 64.2 mmol) was added dropwise and reaction mixture was heated at 40 °C for 16 h. Reaction was monitored by ELSD. After completion, reaction mixture was cooled, diluted with dichloromethane, washed with saturated aqueous sodium bicarbonate solution and water. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-5 % methanol in dichloromethane to afford (2R,3R,4R,5R,6S)-5-acetamido-2-(acetoxymethyl)-6-((3-methoxy-3-oxopropyl)thio)tetrahydro-2H-pyran-3,4-diyl diacetate (**54x-2**) as a colourless viscous liquid. Yield: 5.2 g, 87.39 %; LCMS m/z 450.1 $[M+1]^+$.

To a solution of (2R,3R,4R,5R,6S)-5-acetamido-2-(acetoxymethyl)-6-((3-methoxy-3-oxopropyl)thio)tetrahydro-2H-pyran-3,4-diyl diacetate (**54x-2**, 1.0 eq, 4.0 g, 8.9 mmol) in methanol (40 mL), sodium methoxide (25 % solution in methanol) (0.1 eq, 0.21 mL, 0.89 mmol) was added and reaction mixture was stirred at room temperature for 3 h. After completion, reaction mixture was neutralized with Dowex 50WX8 hydrogen form (200-400 mesh) and filtered through sintered funnel (without celite). The filtrate was concentrated and dried to afford methyl 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoate (**54x-3**) as an off white solid. Yield: 1.7 g, 59.0 %; LCMS m/z 324.0 $[M+1]^+$.

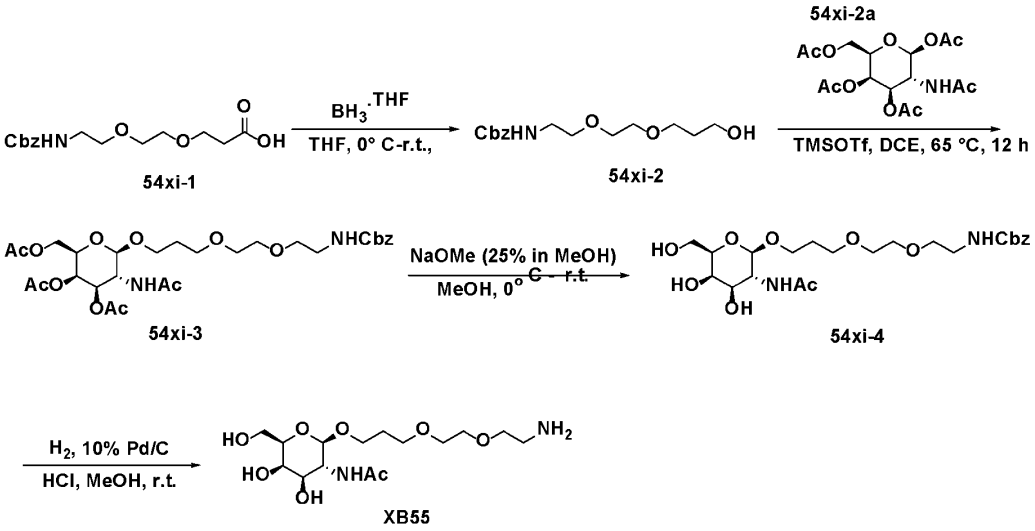
To a solution of methyl 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoate (**54x-3**, 1.0 eq, 2.0 g, 6.19 mmol) in tetrahydrofuran (18 mL), methanol (12 mL) and water (6 mL), lithium hydroxide monohydrate (2.0 eq, 0.519 g, 12.4 mmol) was added and reaction mixture was stirred at room temperature for 2 h. After completion, reaction mixture was concentrated, methanol was added, neutralized with Dowex 50WX8 hydrogen form (200-400 mesh) and filtered through sintered funnel (without celite). The filtrate was concentrated and dried to afford 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoic acid (**54x-4**) as an off white sticky solid. Yield: 2.4 g (Crude); LCMS m/z 310.0 $[M+1]^+$.

To a solution of 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoic acid (**54x-4**, 1.0 eq, 1.1 g, 3.56 mmol) in pyridine (11 mL), acetic anhydride (10.0 eq, 3.36 mL, 35.6 mmol) was added and reaction mixture was stirred at room temperature for 16 h. After completion, reaction mixture was concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-7 % methanol in dichloromethane to afford 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoic acid (**54x-5**) as a colorless viscous liquid. Yield: 1.25 g, 76.74 %; LCMS m/z 436.0 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.24 (s, 1H), 7.88 (d, J = 9.6 Hz, 1H), 5.27 (d, J = 3.2 Hz, 1H), 4.95 (dd, J = 2.4, 10.8 Hz, 1H), 4.66 (d, J = 10.4 Hz, 1H), 4.10-3.96 (m, 4H), 2.85-2.79 (m, 1H), 2.74-2.69 (m, 1H), 2.58 (t, J = 7.2 Hz, 2H), 2.11(s, 3H), 2.00 (s, 3H), 1.90 (s, 3H), 1.77 (s, 3H).

A solution of tert-butyl (5-aminopentyl)carbamate (1.2 eq, 166 mg, 0.821 mmol) and 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)thio)propanoic acid (1.0 eq, 302 mg, 0.694 mmol) and DIPEA (3.0 eq, 0.36 mL, 2.08 mmol) in DMF (3.5 mL) was cooled in an ice bath before adding HATU (1.2 eq, 316 mg, 0.832 mmol) then removing the ice bath. After 45 minutes, the reaction was diluted with water (10 mL) and brine (10 mL) and the products were extracted with EtOAc (2x10 mL). The partitioned aqueous layer was washed with EtOAc (5 mL) and the combined organic layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated under reduced pressure to give crude material that was adsorbed to silica gel for purification by column chromatography (50-100% EtOAc in hexanes) to give (2R,3R,4R,5R,6S)-5-acetamido-2-(acetoxymethyl)-6-((3-((5-((tert-butoxycarbonyl)amino)pentyl)amino)-3-oxopropyl)thio)tetrahydro-2H-pyran-3,4-diyl diacetate. Yield: 319 mg, 74.2 %. LCMS m/z 619.95 $[M+H]^+$.

A solution of [(2R,3R,4R,5R,6S)-5-acetamido-3,4-diacetoxy-6-[3-[5-(tert-butoxycarbonylamino)pentylamino]-3-oxo-propyl]sulfanyl-tetrahydropyran-2-yl]methyl acetate (1.0 eq, 188 mg, 0.303 mmol) in methanol (1.5 mL) was treated with 25% w/w sodium methoxide in methanol (4.0 eq, 0.28 mL, 1.21 mmol). After 1 h, the reaction was cooled in an ice bath, neutralized with 4M HCl in dioxane (4.0 eq, 303 mL, 1.21 mmol) then concentrated under reduced pressure to give crude material. The residue was dissolved in 1:1 MeOH/DCM (3 mL) then treated with 4M HCl in dioxane (4.0 eq, 303 mL, 1.21 mmol). After 1h, the reaction was concentrated under reduced pressure. The residue was dissolved in concentrated NH_4OH then purified by reversed-phase HPLC (3-30% acetonitrile in water + 10 mM NH_4OH) then lyophilized to give 3-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)-N-(5-aminopentyl)propenamide (**XB54**). Yield: 102 mg, 85.5 %. LCMS m/z 394.2 $[M+H]^+$.

(xi) Synthesis of N-((2R,3R,4R,5R,6R)-2-(3-(2-(2-aminoethoxy)ethoxy)propoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB55)



A solution of 3-oxo-1-phenyl-2,7,10-trioxa-4-azatridecan-13-oic acid (**54xi-1**, 7.0 g, 1.0 eq., 22.5 mmol) in dry tetrahydrofuran (70 mL) was cooled to 0° C. To this, borane tetrahydrofuran complex (1M in THF, 112.5 mL, 5.0 eq., 112.5 mmol) was added slowly, and the resulting reaction mixture was stirred at room temperature for 21h. After completion, conc. HCl was added dropwise to quench excess borane complex, and the resulting mixture was stirred for another 30 minutes. Thereafter, the reaction mixture was concentrated under reduced pressure to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 2-5% methanol in dichloromethane to afford benzyl (2-(2-(3-hydroxypropoxy)ethoxy)ethyl)carbamate (**54xi-2**) as colourless viscous liquid. Yield: 2.8 g, 41.88%; LCMS: m/z 298.1 $[M+H]^+$.

To a stirred solution of (2S,3R,4R,5R,6R)-3-acetamido-6-(acetoxymethyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (**54xi-2a**, 1.45 g, 1.0 eq., 3.72 mmol), and benzyl (2-(2-(3-hydroxypropoxy)ethoxy)ethyl)carbamate (**54xi-2**, 1.11 g, 1 eq., 3.72 mmol) in 1,2-dichloroethane (15 mL) at room temperature, was added trimethylsilyl trifluoromethanesulfonate (67.6 µL, 0.1 eq., 372 µmol) drop-wise and the reaction mixture was heated at 65° C for 6h. After completion, the reaction mixture was quenched with triethyl amine and concentrated under reduced pressure to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 2-5% methanol in dichloromethane to afford (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-((3-oxo-1-phenyl-2,7,10-

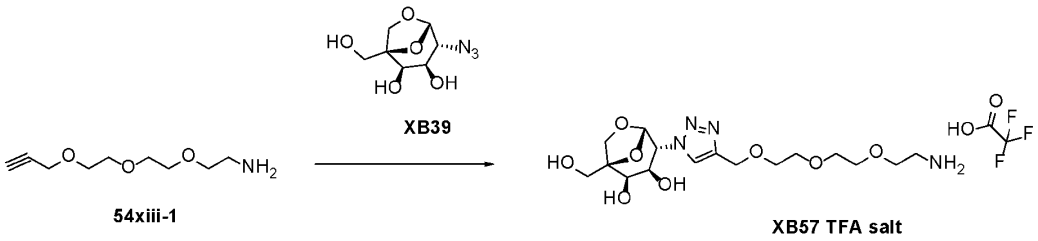
trioxa-4-azatridecan-13-yl)oxy)tetrahydro-2H-pyran-3,4-diyl diacetate (**54xi-3**) as colourless viscous liquid. Yield: 2.08 g, 89.13%; LCMS: m/z 627.0 $[M+H]^+$.

A solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-((3-oxo-1-phenyl-2,7,10-trioxa-4-azatridecan-13-yl)oxy)tetrahydro-2H-pyran-3,4-diyl diacetate (**54xi-3**, 0.350 g, 0.559 mmol) in methanol (4 mL) was cooled to 0° C. To this, sodium methoxide (25% solution in methanol) (0.02 mL, 0.4 eq., 0.223 mmol) was added, and the resulting reaction mixture was stirred at room temperature for 3h. After completion, the reaction mixture was neutralized with Dowex 50WX8 hydrogen form (200-400 mesh) and filtered through sintered funnel (without celite). The filtrate was concentrated to afford benzyl (2-(2-(3-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)propoxy)ethoxy)ethyl)carbamate (**54xi-4**) as colorless viscous liquid. Yield: 0.26 g, 93.0%; LCMS: m/z 501.1 $[M+H]^+$.

To a stirred solution of benzyl (2-(2-(3-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)propoxy)ethoxy)ethyl)carbamate (**54xi-4**, 0.26 g, 0.519 mmol) in methanol (4 mL), 10% palladium on carbon (0.166 g) and conc. HCl (20 µL, 1.0 eq., 0.519 mmol) were added and the reaction mixture was stirred at room temperature under hydrogen gas balloon pressure for 2h. After completion, the reaction mixture was filtered through syringe filter and washed with methanol. The filtrate was concentrated and purified by prep-HPLC (30-50% acetonitrile in water with 0.1 % trifluoroacetic acid). Fractions containing the desired product were combined and lyophilized to dryness to afford N-((2R,3R,4R,5R,6R)-2-(3-(2-(2-aminoethoxy)ethoxy)propoxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (**XB55**) as a colourless semi-solid. Yield: 0.071 g, 37.3%; LCMS: m/z 367.1 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6 with D_2O exchange): δ 4.20 (d, J = 8.4 Hz, 1H), 3.76-3.64 (m, 3H), 3.59-3.55 (m, 4H), 3.58-3.48 (m, 4H), 3.47-3.39 (m, 4H), 3.31-3.28 (t, J = 6.0 Hz, 1H), 2.95 (t, J = 5.2 Hz, 2H), 1.86 (s, 3H), 1.66 (t, J = 6.8, 2H).

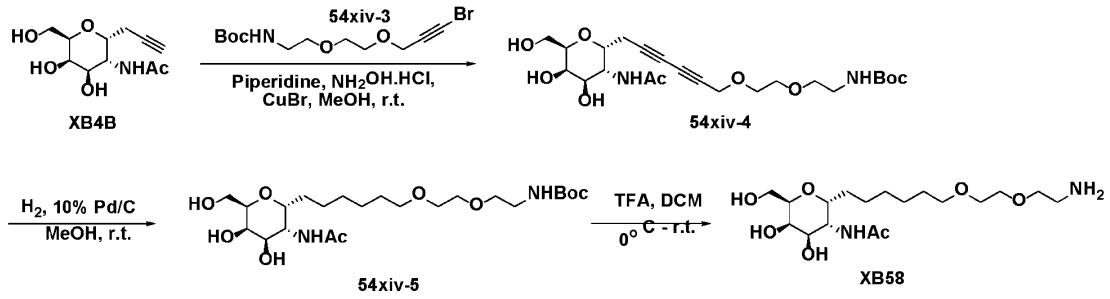
(xii) Synthesis of N-((2S,3R,4R,5R,6R)-2-(5-(2-(2-aminoethoxy)ethoxy)pentyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB56) was prepared by adapting the procedure of XB47. LCMS m/z: 379.4 [M + H]+.

(xiii) Synthesis of (1S,2R,3R,4R,5S)-4-(4-((2-(2-(2-aminoethoxy)ethoxy)ethoxy)methyl)-1H-1,2,3-triazol-1-yl)-1-(hydroxymethyl)-6,8-dioxabicyclo[3.2.1]octane-2,3-diol TFA salt (XB57 TFA salt).



To a solution of 2-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethoxy)ethan-1-amine **(54xiii-1)** (39.7 mg, 0.183 mmol, 1.0 eq.) and (1S,2R,3R,4R,5S)-4-azido-1-(hydroxymethyl)-6,8-dioxabicyclo[3.2.1]octane-2,3-diol **(XB39)** (34.4 mg, 0.184 mmol, 1.0 eq.) in 0.4 mL of dimethyl sulfoxide was added tetrakis(acetonitrile)copper(I) hexafluorophosphate(67.4 mg, 0.181 mmol, 0.99 eq) as a solid in one portion. The mixture was stirred under nitrogen atmosphere at ambient temperature for approximately 15 min until completion. The reaction mixture was diluted with water, which formed a precipitate, then 2 drops of trifluoroacetic acid was added to clear the solution. The product was isolated from diluted mixture by preparatory HPLC, eluting with 1-20% acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford trifluoroacetic acid salt of Compound **XB57** as a clear oil. Yield: 47.9 mg (50%); LCMS m/z 405.3 [M+1]+.

(xiv) Synthesis of N-((2R,3R,4R,5R,6R)-2-(6-(2-(2-aminoethoxy)ethoxy)hexyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (XB58)



A solution (**A**) of *N*-((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-(prop-2-yn-1-yl)tetra hydro-2H-pyran-3-yl)acetamide (**XB4B**, 0.4 g, 1.0 eq., 1.4 mmol), piperidine (0.298 g, 2.5 eq., 3.49 mmol), copper(I) bromide (0.02 g, 0.1 eq., 0.140 mmol) and hydroxylamine hydrochloride (0.0194 g, 0.2 eq., 0.28 mmol) in methanol (8 mL) was purged with N_2 for 30 minutes. Another solution (**B**) of tert-butyl (2-(2-((3-bromoprop-2-yn-1-yl)oxy)ethoxy)ethyl)carbamate (**54xiv-3**, 0.45 g, 1.0 eq., 1.4 mmol) in methanol (4 mL) was purged with N_2 for 30 minutes. Solution (**B**) was added slowly to solution (**A**) through syringe over a period of 15 minutes, and the reaction mixture was stirred at room temperature for 12 h. After completion, methanol was removed under reduced pressure and the crude product obtained was poured into ice cold water (10 mL), and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulphate, filtered and concentrated to afford tert-butyl (2-(2-((6-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)hexa-2,4-diyn-1-yl)oxy)ethoxy)ethyl)carbamate (**54xiv-4**) as colourless viscous liquid. Yield: 0.18 g (Crude); LCMS: m/z 385.2 [M+H]+.

To a solution of tert-butyl (2-(2-((6-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)hexa-2,4-diyn-1-yl)oxy)ethoxy)ethyl)carbamate (**54xiv-4**, 0.10 g, 1.0 eq., 0.206 mmol) in methanol (3 mL), 10 % palladium on carbon (0.070 g) was added and reaction mixture was stirred under hydrogen gas atmosphere at room temperature for 3h. After completion (monitored by LCMS., reaction mixture was filtered through syringe filter, filtrate was concentrated to afford tert-butyl (2-(2-((6-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)hexyl)oxy)ethoxy)ethyl)carbamate (**54xiv-5**) as a colorless viscous liquid. Yield: 0.090 g; LCMS: m/z 493.15 $[M+H]^+$.

A solution of tert-butyl (2-(2-((6-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)hexyl)oxy)ethoxy)ethyl)carbamate (**54xiv-5,** 1.0 eq, 0.090 g, 0.182 mmol) in dichloromethane (2 mL) was cooled at 0 °C, trifluoroacetic acid (0.4 mL) was added and reaction mixture was stirred at room temperature for 3h. After completion, reaction mixture was concentrated to get crude which was purified by prep HPLC (20-50 % acetonitrile in water with 0.1 % trifluoroacetic acid). Fractions containing the desired product were combined and lyophilized to dryness to afford N-((2R,3R,4R,5R,6R)-2-(6-(2-(2-aminoethoxy)ethoxy)hexyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (**XB58**) as a brown solid. Yield: 0.015 g, 20.25 %; LCMS: m/z 393.2 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-d_6 with D_2O) δ 3.99-3.94 (m, 1H), 3.82-3.79 (m, 1H), 3.70 (bs, 1H), 3.51-3.49 (m, 7H), 3.37 (t, J = 6.4 Hz, 2H), 2.95 (t, J = 4.8 Hz, 2H), 1.8 (s, 3H), 1.53-1.44 (m, 4H), 1.24-1.15 (m, 6H).

Example 2: Preparation of Cys Reactive ASGPR Targeting Compounds

The following section provides details for the preparation of example trivalent and divalent Cys reactive ASGPR target compounds. Table 8 illustrates various trivalent Cys reactive ASGPR target compounds synthesized using the starting amines indicated in table 14 via the general methods described below (Methods A-C).

Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds

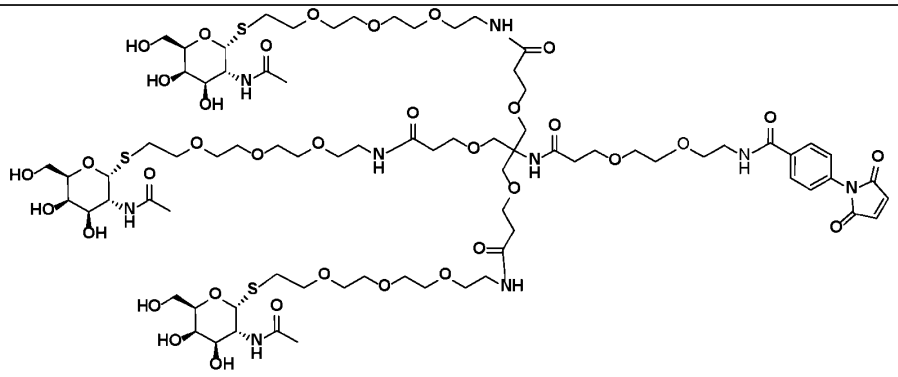
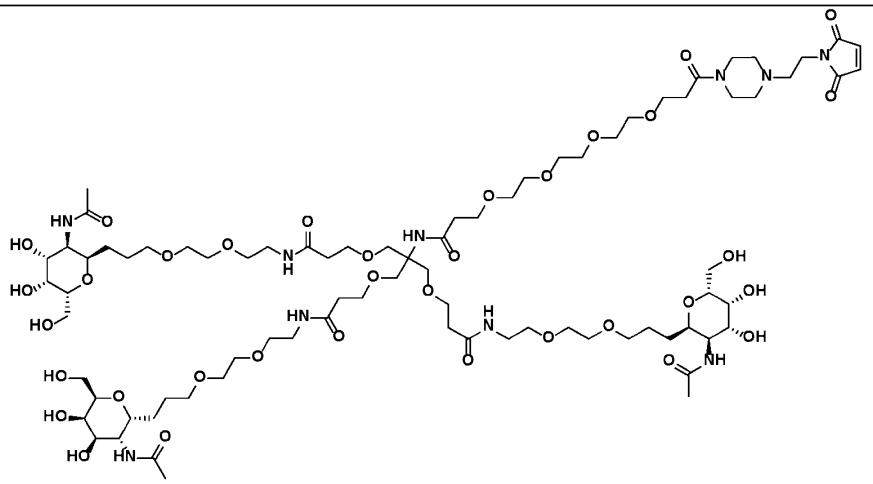
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1246:** **Starting Amine:** XB50, **Method:** Method B* **LCMS:** 940.5 [M+2H]++
 **1248:** **Starting Amine:** XB48, **Method:** Method A **LCMS:** 1804.7 [M+H]+

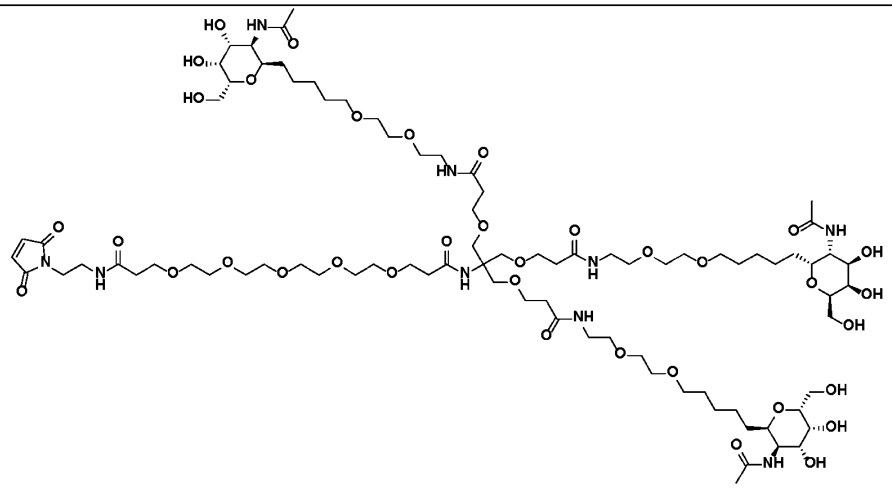
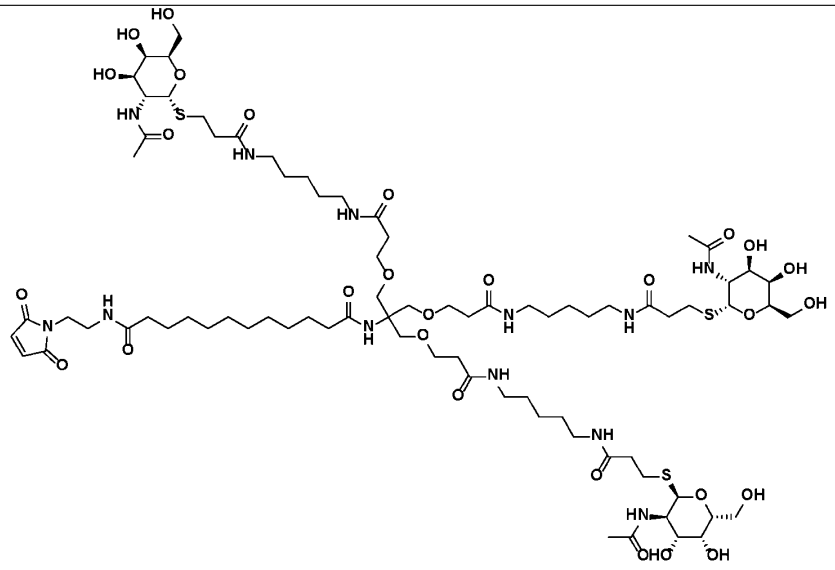
Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1250:** **Starting Amine:** XB47, **Method:** Method C **LCMS:** 1861.7 [M + H]+
 **1225A:** **Starting Amine:** XB49, **Method:** Method C **LCMS:** 1798.7 $[M + H]^+$

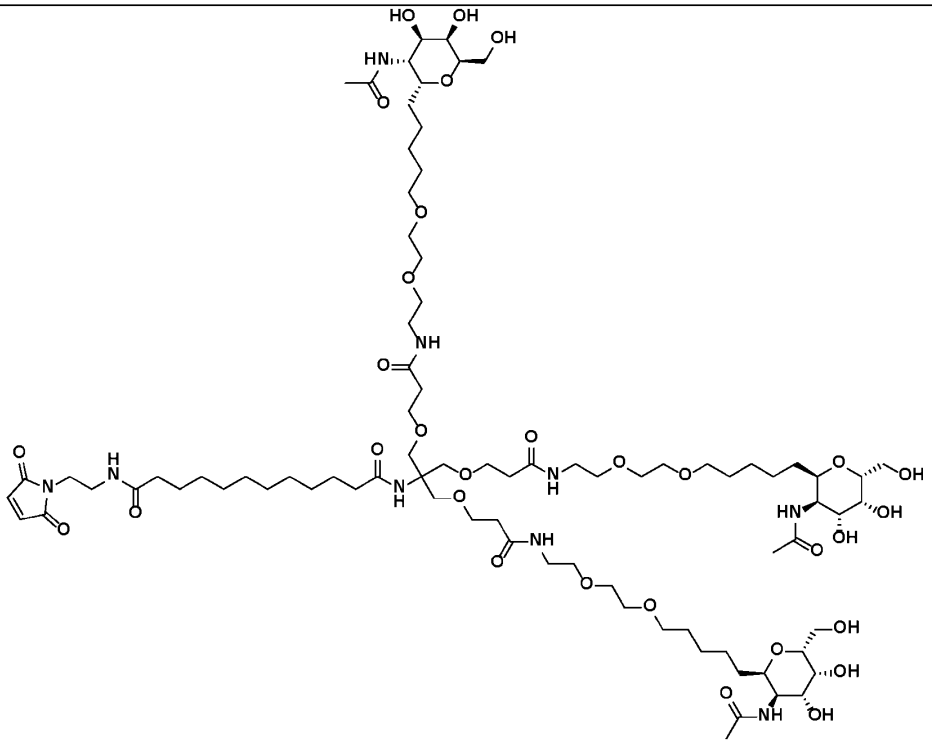
Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1251:** **Starting Amine:** XB47, **Method:** Method C **LCMS:** 1753.7 $[M + H]^+$

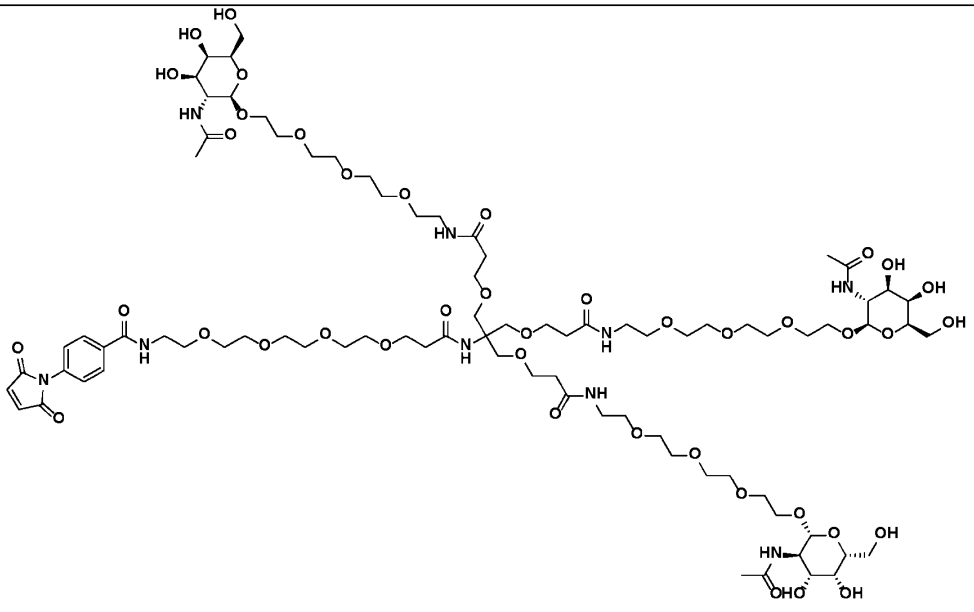
Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1252:** **Starting Amine:** XB53, **Method:** Method B **LCMS:** 1915.1 [M+H]+

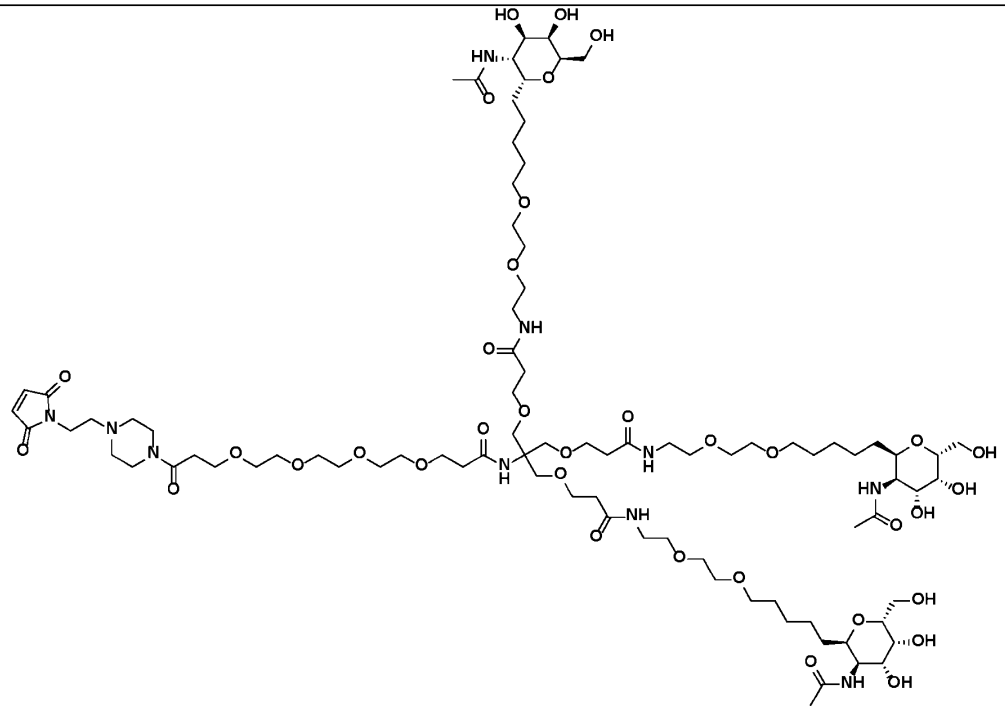
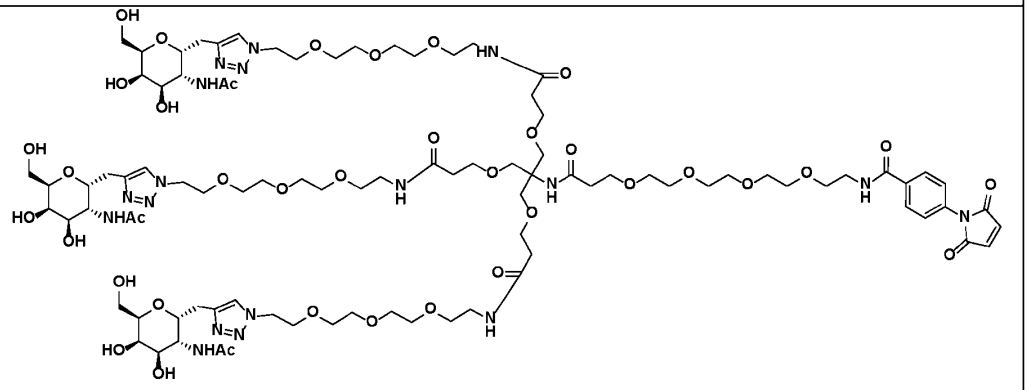
Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1249:** **Starting Amine:** XB47, **Method:** Method A **LCMS:** 1886.8 $[M + H]^+$
 **1258:** **Starting Amine:** XB46, **Method:** Method B **LCMS:** 1058.5 [M+2H]++

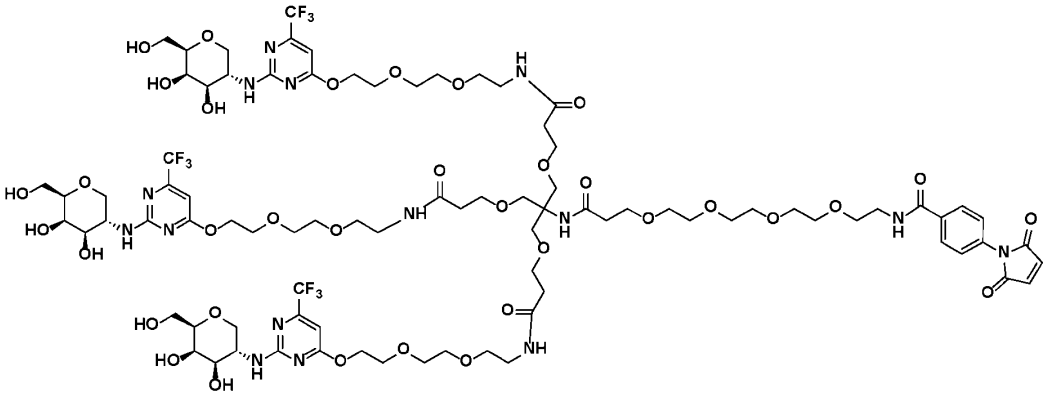
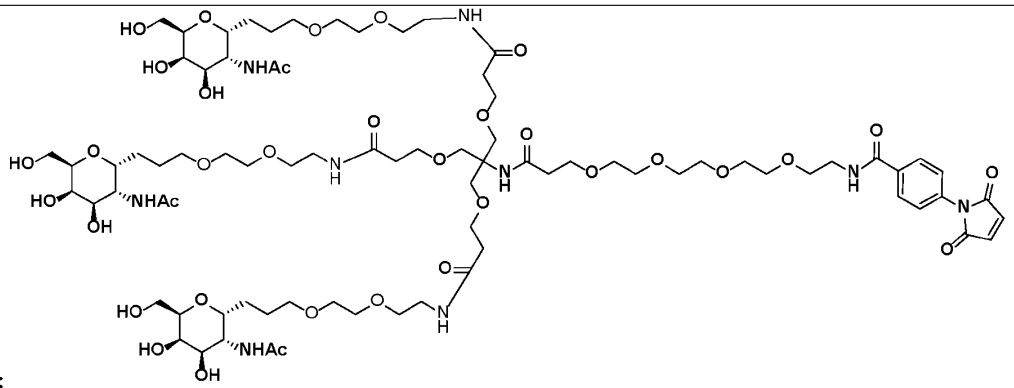
Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
1921:  **Starting Amine:** XB51, **Method:** Method B **LCMS:** 1050.2 [M+2H]++
 **1253:** **Starting Amine:** XB48, **Method:** Method B **LCMS:** 1781.7 [M+H]+

Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds

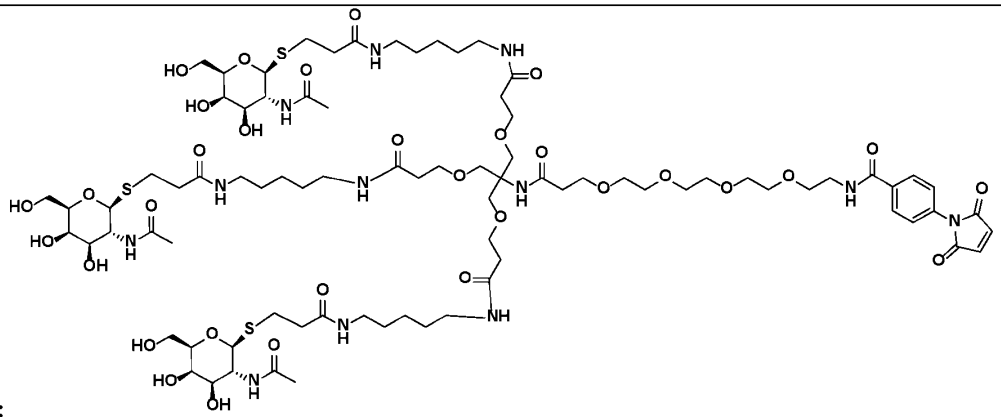
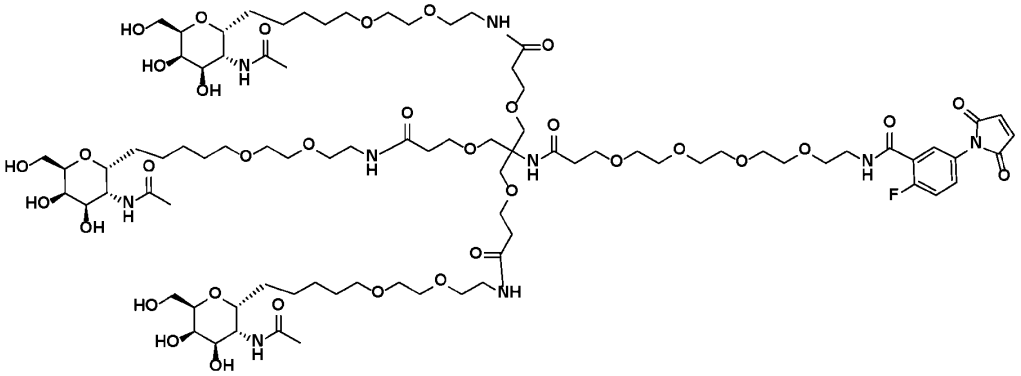
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1255:** **Starting Amine:** XB54, **Method:** Method B **LCMS:** 1910.6 [M+H]+
1257:  **Starting Amine:** XB47, **Method:** Method B* **LCMS:** 1883.7 $[M + H]^+$

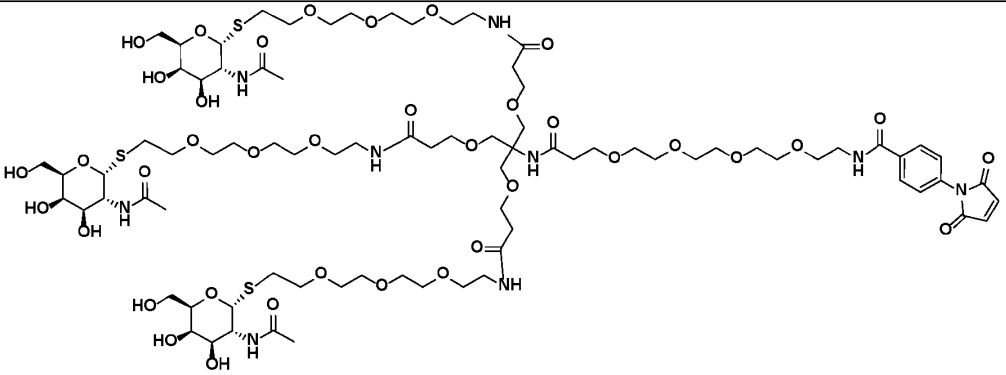
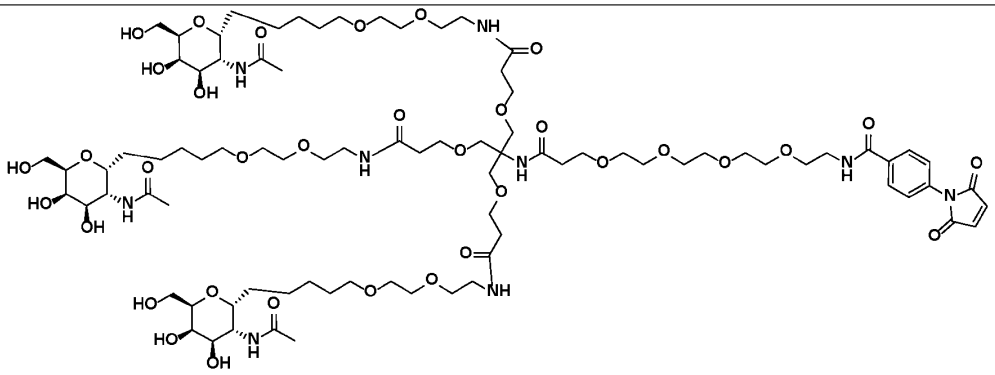
Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1247:** **Starting Amine:** XB50, **Method:** Method B **LCMS:** 1967.3 $[M + H]^+$
 **1254:** **Starting Amine:** XB47, **Method:** Method B **LCMS:** 1865.6 $[M + H]^+$

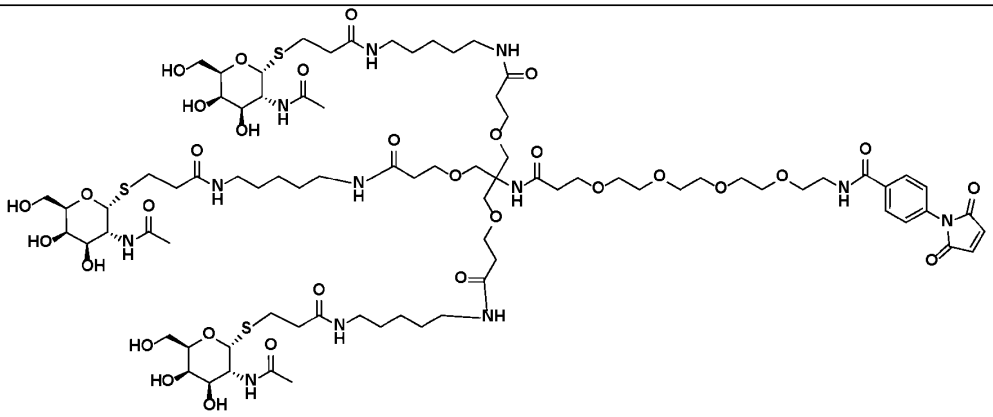
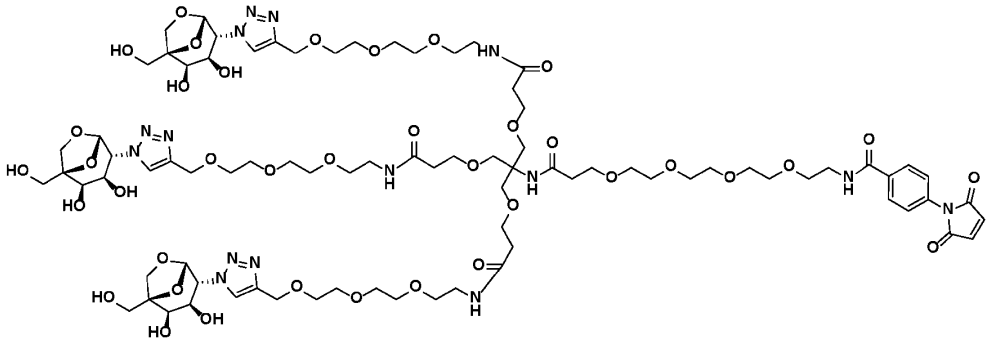
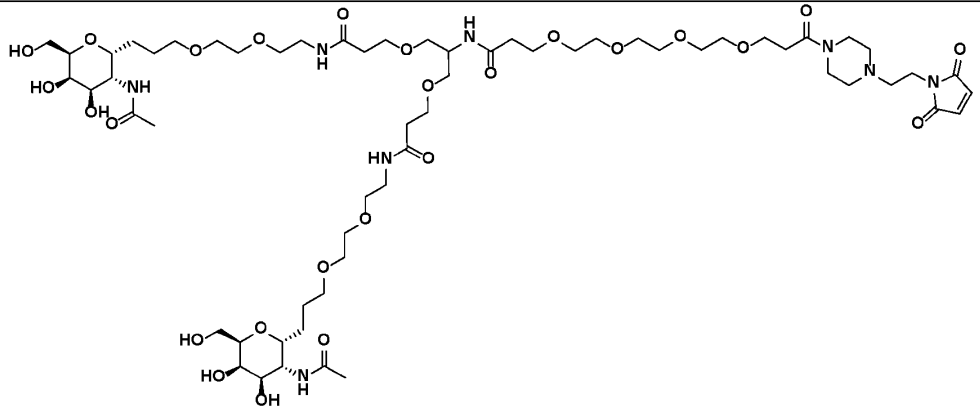
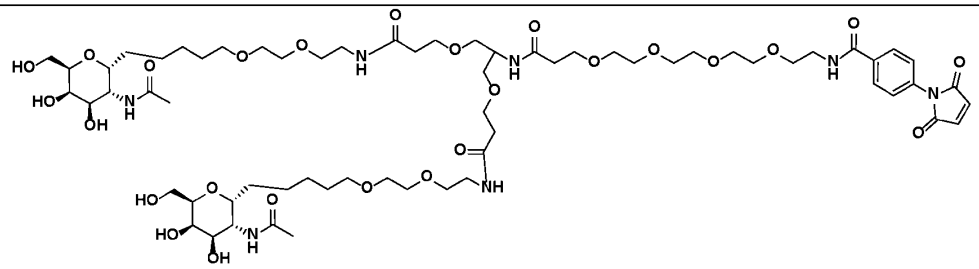
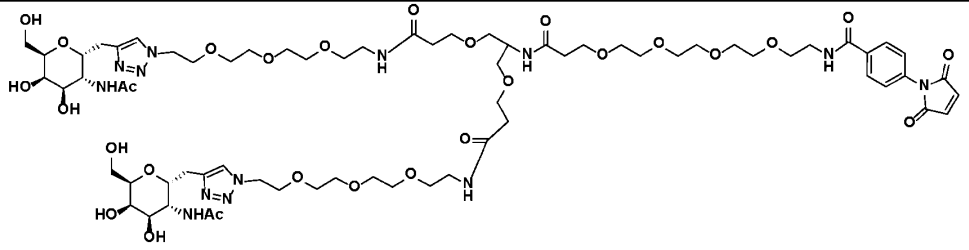
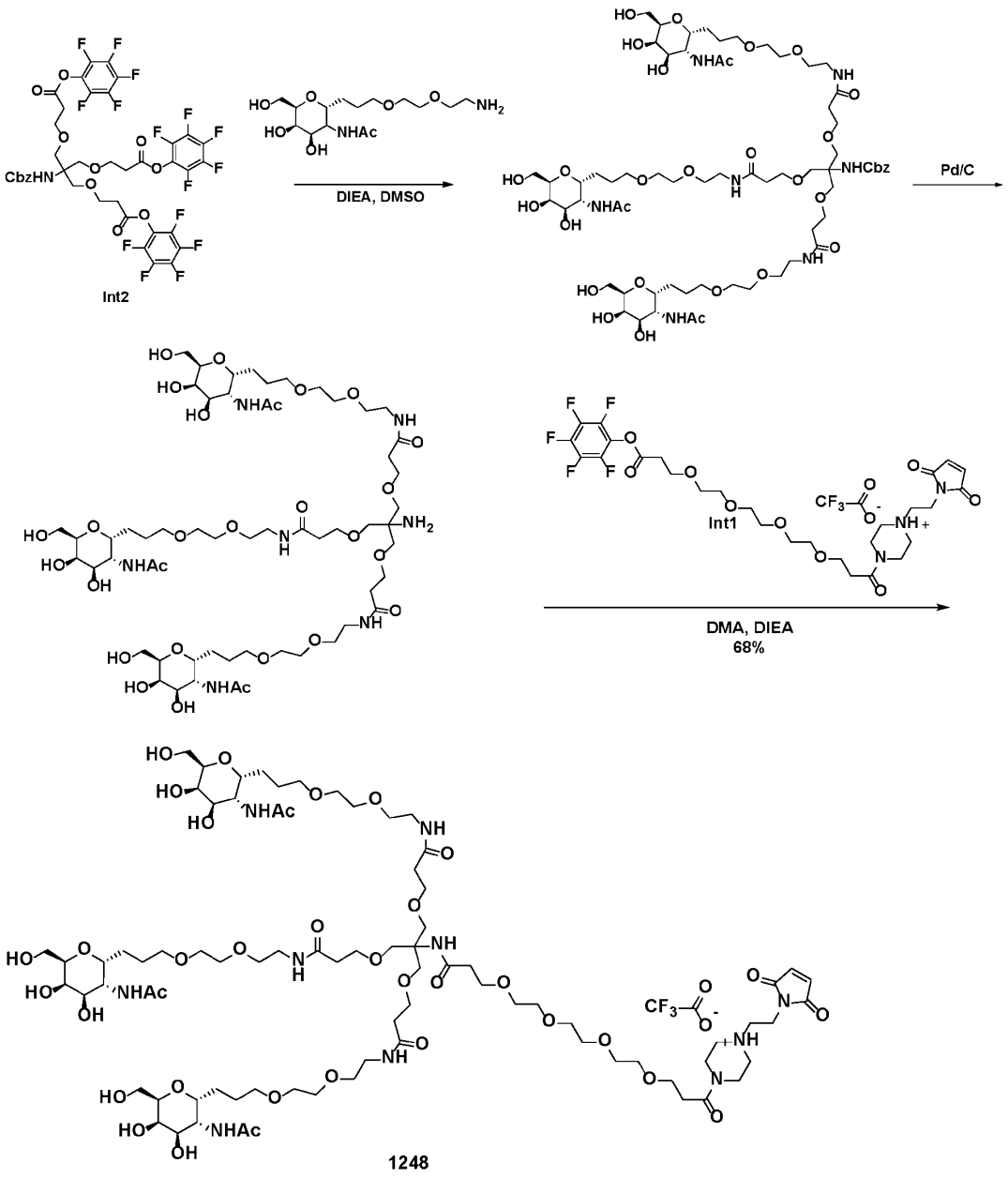
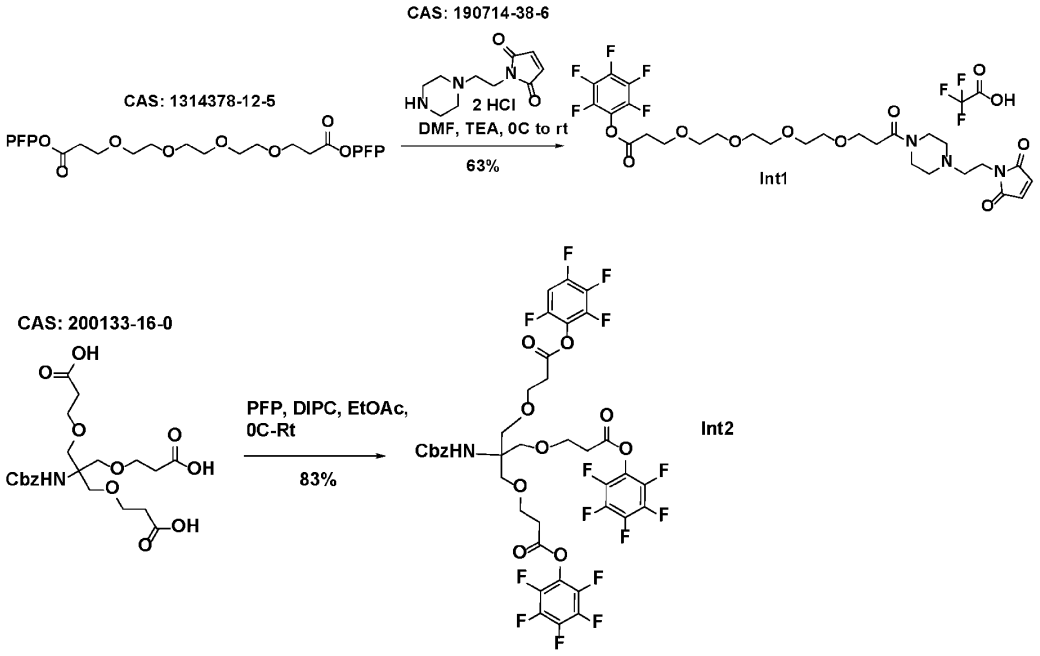
Table 8: Synthesis of trivalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1259:** **Starting Amine:** XB47, **Method:** Method B **LCMS:** 1908.5 $[M - H]^-$ (Neg Mode)
1915A:  **Starting Amine:** XB57, **Method:** Method B **LCMS:** 1943.5 $[M + H]^+$

Table 9 illustrates various divalent Cys reactive ASGPR target compounds synthesized via the general methods described herein.

Table 9: Synthesis of divalent Cys reactive ASGPR targeting compounds
Compound #, Starting amine used, synthesis method and LCMS m/z observed
 **1219:** **Starting Amine:** XB48, **Method:** Method A **LCMS:** 1368.1 $[M + H]^+$
 **1221:** **Starting Amine:** XB47, **Method:** Method B **LCMS:** 1402.6 $[M + H]^+$
 **1237:** **Starting Amine:** XB46, **Method:** Method B **LCMS:** 1568.6 $[M + H]^+$

Method A (e.g., for the synthesis of Compound 1248)





Synthesis of perfluorophenyl 16-(4-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)piperazin-1-yl)-16-oxo-4,7,10,13-tetraoxahexadecanoate, TFA salt(**Int1**): To a suspension of 1-(2-piperazin-1-ylethyl)pyrrole-2,5-dione;dihydrochloride (1.00 eq, 202 mg, 0.716 mmol) in 1.5mL of DMF was added Bis-PEG4-PFP ester (1.50 eq, 673 mg, 1.07 mmol) in 1 mL DMF, the mixture was cooled to 0°C and triethylamine (3.00 eq, 0.30 mL, 2.15 mmol) was added dropwise - mixture remains cloudy, partial slurry - stirred at 0°C for 20 min and then let warm to room temp and stirred for 30 min. The mixture was cooled to 0°C, acidified with 165uL TFA (3 eq) in approx. 0.50 mL water, diluted with CH3CN, filtered and purified by preparative HPLC on C18 column (30x250mm) eluting with a gradient of 15-75-100% CH3CN/water + 0.1 %TFA. Collected 343 mg of the desired product as a clear oil after lyophilization of fractions, 63% yield. HPLC: 100% by ELSD, >99% @ 254nm [5-99 CH3CN/water+0.1% TFA over 8 min., 0.9 mL/min, Agilent-poroshell 120, C18, 2.7 um, 50x3 mm] LCMS 652.5 [M+1], [10-100 CH3CN/water+0.1% FA over 7 min., 0.9 ml/min, Agilent, poroshell120,C18, 2.1 mmx50mm, 2.7um]; 1H NMR (400 MHz, CDCl3) δ 6.75 (s, 2H), 3.91 (m, 6H), 3.79 (t, J = 5.8 Hz, 2H), 3.71 - 3.56 (m, 14H), 3.39 (t, J = 5.6 Hz, 2H), 3.39 (brs, 4H), 2.97 (t, J = 6.1 Hz, 2H), 2.67 (br s, 2H). LCMS: 652.5 [M+H]+.

Synthesis of bis(perfluorophenyl) 3,3'-((2-(((benzyloxy)carbonyl)amino)-2-((3-oxo-3-(perfluorophenoxy)propoxy)methyl)propane-1,3-diyl)bis(oxy))dipropionate (**Int2**): A solution of 3,3'-((2-(((benzyloxy)carbonyl)amino)-2-((2-carboxyethoxy)methyl)propane-1,3-diyl)bis(oxy))dipropionic acid (1.0 eq, 10.0 g, 21.2 mmol) in ethyl acetate (100 mL) was cooled at 0 °C, 2,3,4,5,6-pentafluorophenol

(3.0 eq, 11.7 g, 63.6 mmol) and N,N'-diisopropylcarbodiimide (4.0 eq, 13.3 mL, 84.8 mmol) were added and reaction mixture was stirred at room temperature for 16 h. After completion, reaction mixture was filtered through sintered funnel (without celite) and filtrate was concentrated to a crude residue, which was purified by column chromatography using silica gel (100-200 mesh) and 0-15 % ethyl acetate in hexane to afford bis(perfluorophenyl) 3,3'-((2-(((benzyloxy)carbonyl)amino)-2-((3-oxo-3-(perfluorophenoxy)propoxy)methyl)propane-1,3-diyl)bis(oxy))dipropionate **(Int2)** as an off white solid. Yield: 17.0 g, 82.66 %; LCMS m/z 970.39 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34-7.26 (m, 5H), 6.58 (s, 1H), 4.95 (s, 2H), 3.75 (t, J = 5.6 Hz, 6H), 3.59 (s, 6H), 2.98 (t, J = 5.6 Hz, 6H).

Synthesis of Compound **1248**:

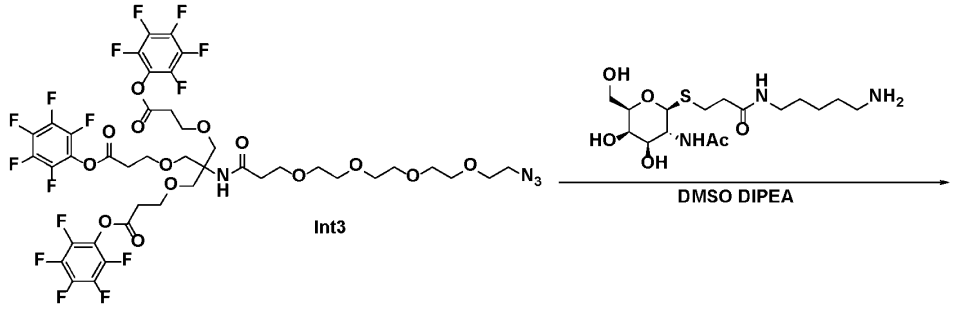
A solution of N-[(2R,3R,4R,5R,6R)-2-[3-[2-(2-aminoethoxy)ethoxy]propyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (3.30 eq, 346 mg, 0.987 mmol) and (2,3,4,5,6-pentafluorophenyl) 3-[2-(benzyloxycarbonylamino)-3-[3-oxo-3-(2,3,4,5,6-pentafluorophenoxy)propoxy]-2-[[3-oxo-3-(2,3,4,5,6-pentafluorophenoxy)propoxy]methyl]propoxy]propanoate (1.00 eq, 290 mg, 0.299 mmol) in DMSO (1.4955 mL) was treated with Diisopropylethylamine (DIPEA) (3.00 eq, 156 uL, 0.897 mmol). After 90 minutes, the reaction was diluted with water (500 uL), formic acid (150uL) and methanol (350uL) and the reaction was purified by RPHPLC (3-50% CAN in water w/0.1% FA) to give benzyl N-[2-[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]-1,1-bis[[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]methyl]ethyl]carbamate as a white solid. Yield: 405 mg, 92%. LCMS m/z 1469.1 $[M+H]^+$.

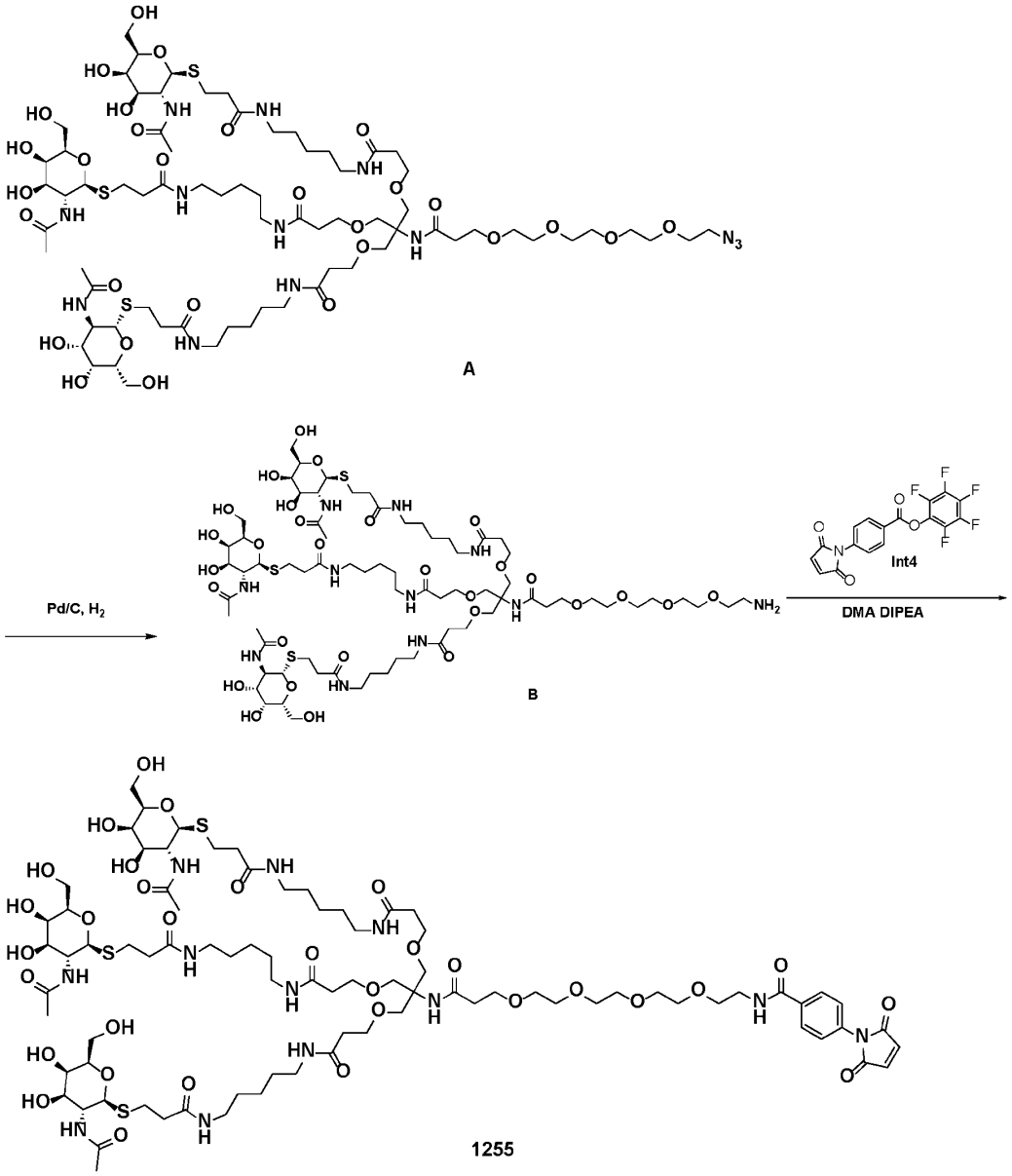
A mixture of benzyl N-[2-[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]-1,1-bis[[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]methyl]ethyl]carbamate (1.00 eq, 390 mg, 0.266 mmol) and 10% Pd/C, Evonik Noblyst (0.700 eq, 396 mg, 0.186 mmol) in methanol (5.3 mL) was stirred vigorously under an atmosphere of nitrogen gas. After 1h, the reaction was filtered through celite, the filter cake was washed with methanol (50mL) and the filtrate was concentrated under reduced pressure to a syrup. The syrup (~1.8mL) was filtered (some darkness of carbon observed) then dripped into EtOAc (8 mL) but a sticky ppt was formed so the slurry was concentrated under reduced pressure to give N-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethyl]-3-[3-[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]-2-[[3-[2-[2-[3-

[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]methyl]-2-amino-propoxy]propenamide as a white solid. The crude material was used in the next step without further purification. Yield: 326 mg, 92%. LCMS m/z 1335.1 $[M+H]^+$.

A mixture of N-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethyl]-3-[3-[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]-2-[[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]methyl]-2-amino-propoxy]midazolidi (1.00 eq, 250 mg, 0.187 mmol) and (2,3,4,5,6-pentafluorophenyl) 3-[2-[2-[2-[3-[4-[2-(2,5-dioxopyrrol-1-yl)ethyl]piperazin-1-yl]-3-oxo-propoxy]ethoxy]ethoxy]ethoxy]propanoate;2,2,2-trifluoroacetic acid (1.20 eq, 172 mg, 0.225 mmol) in DMF (1.8734 mL) was treated with diisopropylethylamine (4.00 eq, 131 uL, 0.749 mmol) and the reaction was stirred at room temperature. After 19h, another charge of PFP-ester was added (0.24 eq, 34 mg). After 50h, another charge of PFP-ester (0.26 eq, 40mg) was added. After 68h, the reaction was diluted with DMSO (500uL) then purified by RPHLC (2% then 10% then 10-20% acetonitrile in water w/0.1% TFA) to give Compound **1248** as a white solid. Yield: 245mg, 68%. LCMS m/z 902.3 $[M+2H]^{++}$. HPLC: 99% based on ELSD.

Method B (e.g., for the synthesis of Compound 1255)





Synthesis of Compound **A:** A solution of perfluorophenyl 1-azido-15-oxo-17,17-bis((3-oxo-3-(perfluorophenoxy)propoxy)methyl)-3,6,9,12,19-pentaoxa-16-azadocosan-22-oate (**Int3**, 1.0 eq, 72.0 mg, 0.0649 mmol) and DIPEA (9.0 eq, 102 mL, 0.584 mmol) in DMSO (649 mL) was treated with 3-[(2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]sulfanyl-N-(5-aminopentyl)midazolidi (3.3 eq, 84.3 mg, 0.214 mmol). After 2 h, the reaction was purified by reversed-phase HPLC (10-50% acetonitrile in water w/0.1% TFA) to give ***(Cmpd A)*** N-[2-[3-[5-[3-

[(2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]sulfanylpropanoylamino]pentylamino]-3-oxo-propoxy]-1,1-bis[[3-[5-[3-[(2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]sulfanylpropanoylamino]pentylamino]-3-oxo-propoxy]methyl]ethyl]-3-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethoxy]midazolidi. Yield: 81 mg, 72%. LCMS m/z 1737.4 $[M+H]^+$.

Synthesis of Compound **B**: A mixture of ***Cmpd A*** (1.0 eq, 42.0 mg, 0.0242 mmol), 10% Pd/C, Evonik Noblyst (0.84 eq, 43.4 mg, 0.0204 mmol) and acetic acid (1.67 mL) was placed under a hydrogen atmosphere via balloon for 1 h. The reaction was filtered through celite then a 0.2 mm syringe filter then concentrated under reduced pressure. The residue was concentrated from water then from MeCN before leaving under high vacuum to give ***Cmpd B***. Yield: 40 mg, 93%. LCMS m/z 1710.8 $[M+H]^+$.

Synthesis of Compound **1255**: A cold solution of perfluorophenyl 4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)benzoate(1.2 eq, 5.2 mg, 0.0136 mmol) in DMA (100 mL) was added to a cold solution of ***Cmpd B*** (1.0 eq, 20.0 mg, 0.0113 mmol) and DIPEA (4.00 eq, 7.9 mL, 0.0452 mmol) in DMA (100 mL) being cooled in an ice bath. After 90 minutes, the reaction was diluted with DMSO, water and some methanol for rinsing then the crude solution was purified by reversed-phase HPLC (5-50% MeCN in water w/0.1% formic acid) to give ***Compound 1255***. Yield: 10 mg, 48%. LCMS m/z 1910.6 $[M+H]^+$.

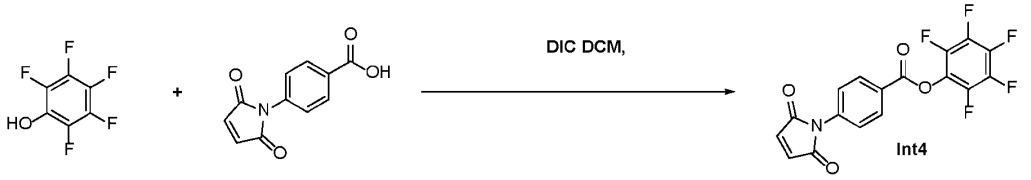
Synthesis of Int3: A solution of di-tert-butyl 3,3'-((2-amino-2-((3-(tert-butoxy)-3-oxopropoxy)methyl)propane-1,3-diyl)bis(oxy))dipropionate (452 mg, 0.894 mmol, 1.00 eq.) and 2,5-dioxopyrrolidin-1-yl 1-azido-3,6,9,12-tetraoxapentadecan-15-oate (356 mg, 0.916 mmol, 1.02 eq.) in 1 mL acetonitrile was stirred under nitrogen atmosphere and heated at 45 °C for 2 days until minimal starting material remained. The reaction mixture was diluted with dichloromethane and evaporated onto silica, then purified by flash column chromatography column, eluting with 0-100% ethyl acetate/dichloromethane. Fractions containing product were concentrate and dried further under high vacuum at ambient temperature to afford tert-butyl 1-azido-17,17-bis((3-(tert-butoxy)-3-oxopropoxy)methyl)-15-oxo-3,6,9,12,19-pentaoxa-16-azadocosan-22-oate as a clear thick syrup. Yield: 559 mg (78%); LCMS m/z 779.2 [M+H]+.

To tert-butyl 1-azido-17,17-bis((3-(tert-butoxy)-3-oxopropoxy)methyl)-15-oxo-3,6,9,12,19-pentaoxa-16-azadocosan-22-oate (509 mg, 0.654 mmol) was added 10 mL of a pre-mixed solution of 30% trifluoroacetic acid in dichloromethane. The mixture stirred at ambient temperature for 3.5 hrs., until consumption of starting material. The solvent was evaporated to residue under a stream of nitrogen, dried further under high vacuum at ambient temperature, then dissolved in 20% water/acetonitrile and lyophilized to dryness to afford 1-azido-17,17-bis((2-carboxyethoxy)methyl)-15-oxo-3,6,9,12,19-

pentaoxa-16-azadocosan-22-oic acid as a thick dark yellow color syrup. Yield: 424 mg (99%); LCMS m/z 611.3 [M+1]+, 609.4 [M-1]-.

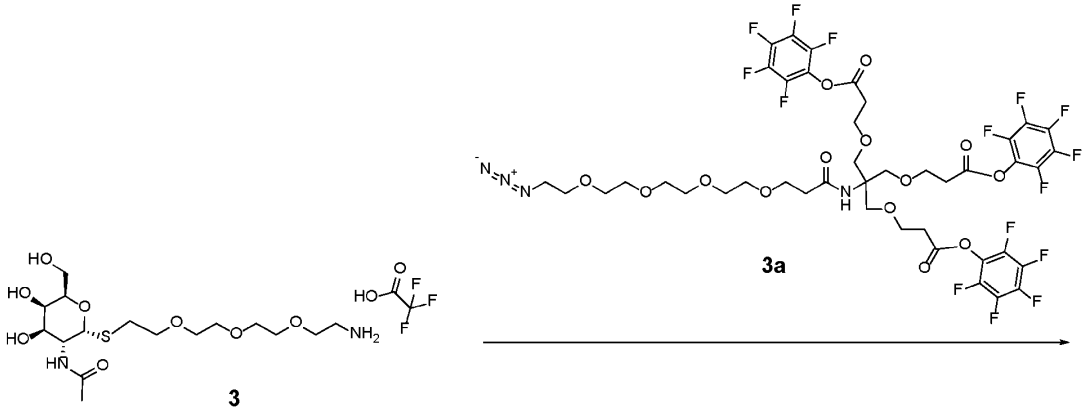
A solution of 1-azido-17,17-bis((2-carboxyethoxy)methyl)-15-oxo-3,6,9,12,19-pentaoxa-16-azadocosan-22-oic acid (81.3 mg, 0.133 mmol, 1.0 eq.) and 2,3,4,5,6-pentafluorophenol (91.8 mg, 0.499 mmol, 3.7 eq.) in 1.5 mL of dichloromethane was added *N,N'*-diisopropylcarbodiimide (90 mL, 0.581 mmol, 4.4 eq.). The mixture stirred at ambient temperature for 1 hr., until consumption of starting material. The reaction mixture was diluted with dichloromethane, evaporated onto silica, then purified by flash column chromatography column, eluting with 0-100% ethyl acetate/hexanes. Fractions containing product were concentrate and dried further under high vacuum at ambient temperature to afford **Int3** as a clear thick syrup. Yield: 104 mg (67%); LCMS m/z 1109.1 [M+H]+.

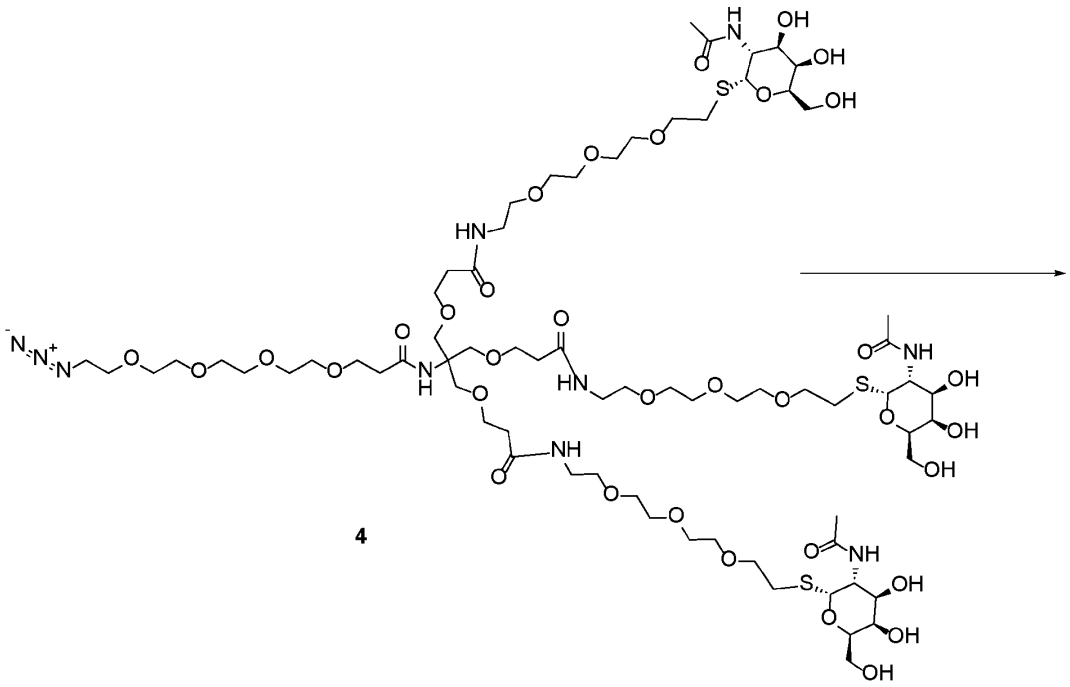
Synthesis of **Int 4**:



Synthesis of (2,3,4,5,6-pentafluorophenyl) 4-(2,5-dioxopyrrol-1-yl)benzoate **Int 4**: To a mixture of 2,3,4,5,6-Pentafluorophenol (1.20 eq, 300 mg, 1.63 mmol) and 4-Maleimidobenzoic acid (1.00 eq, 295 mg, 1.36 mmol) in DCM (6 mL) was added 1,3-diisopropylcarbodiimide (1.50 eq, 0.32 mL, 2.04 mmol). The mixture was stirred at room temperature for 2h and filtered. The filtrate was concentrated and purified by column (0- 25% EtOAc/hexane) to give a white solid (**Int4**, 449 mg, yield: 86%). 1H NMR (400 MHz, CDCl3) δ 8.37 - 8.28 (m, 2H), 7.72 - 7.62 (m, 2H), 6.95 (s, 2H).

Synthesis of Compound **1247**:





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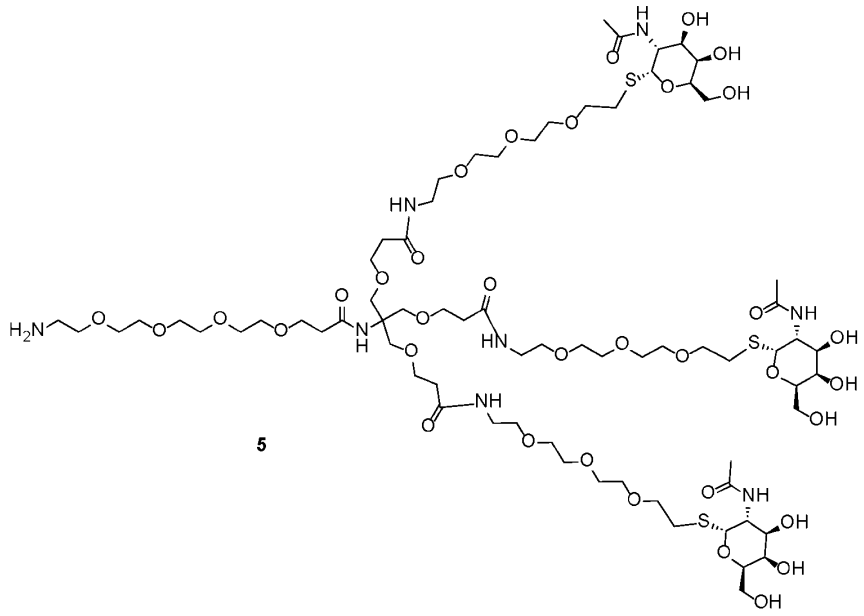

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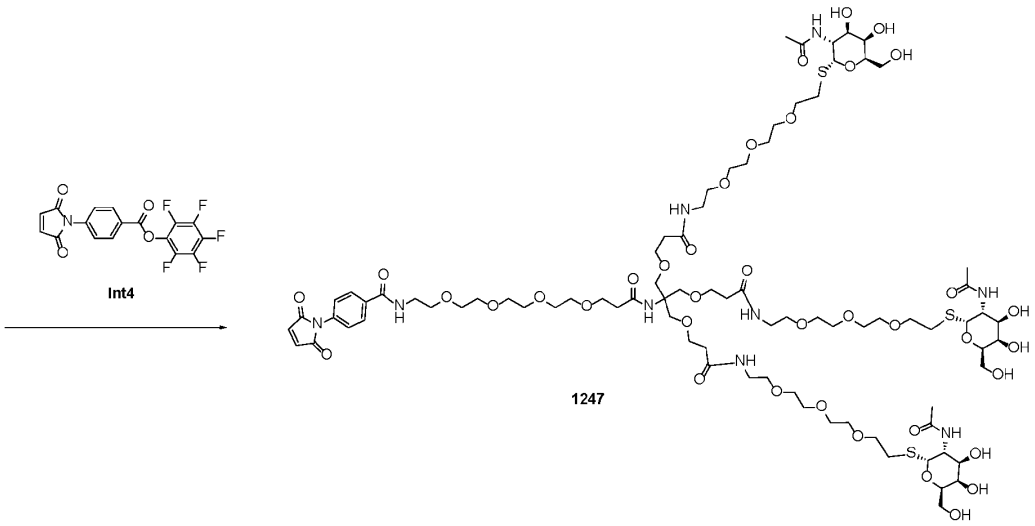


To a solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-((2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-yl)thio)tetrahydro-2H-pyran-3,4-diyl diacetate (165 mg, 0.258 mmol) in 2 mL of methanol, stirring under nitrogen atmosphere at 0-5C, was added sodium methoxide (25% w/w in methanol) (334.8 mg, 1.55 mmol, 6.0 eq) diluted in 1.5 mL of methanol. The reaction mixture stirred at ambient temperature for 15 minutes until completion, at which time 2.0 mL of 1N aqueous hydrochloric acid was added slowly to achieve approximate final pH of 1-2. The reaction mixture was concentrated to approximately 2 mL and purified by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford tert-butyl (2-(2-(2-(2-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)ethoxy)ethoxy)ethoxy)ethyl)carbamate as white solid. Yield: 91.4 mg (69%); LCMS m/z 512.0 [M+1]+, 413.2 [M-Boc+1]+.

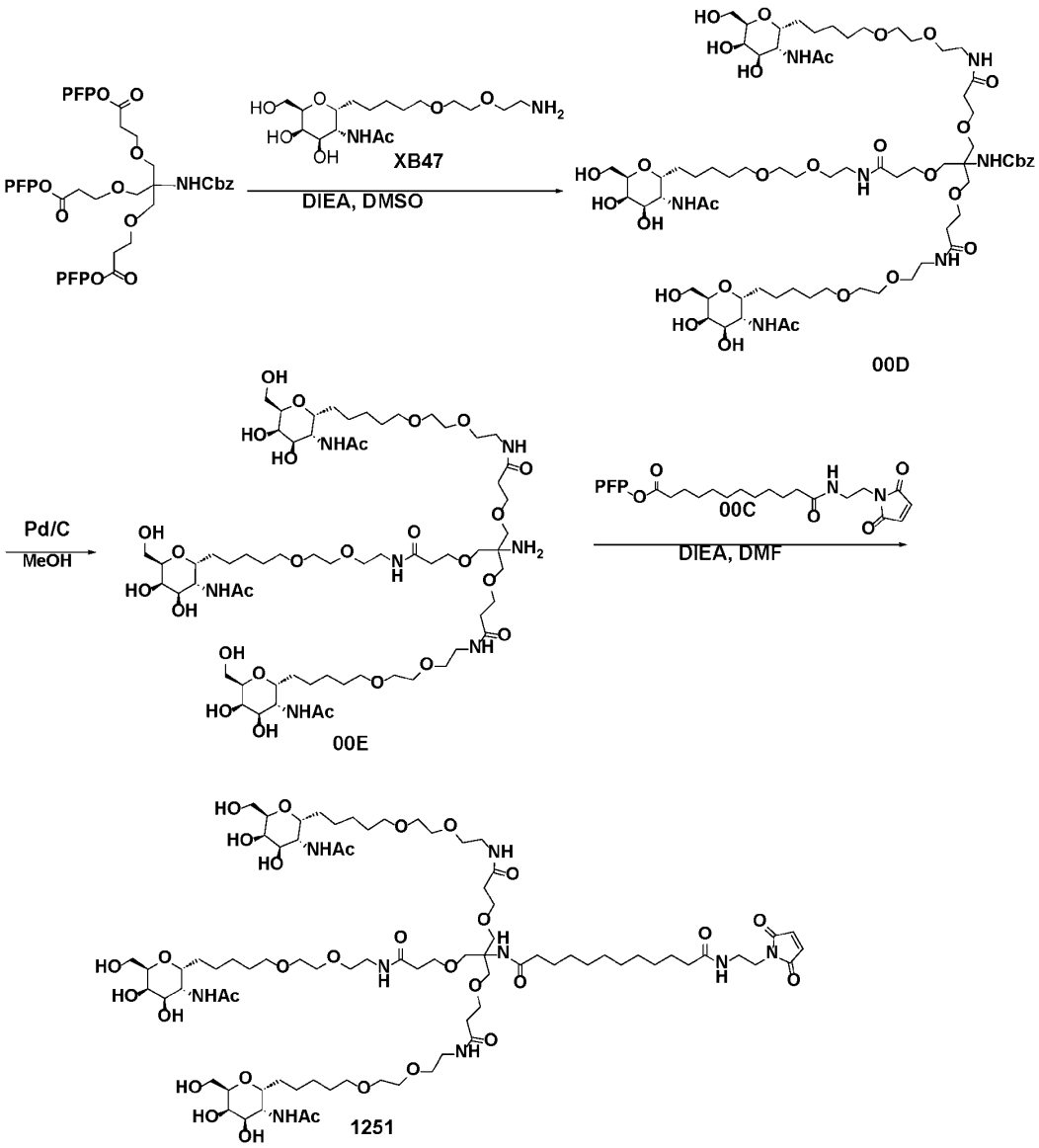
To tert-butyl (2-(2-(2-(2-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)thio)ethoxy)ethoxy)ethoxy)ethyl)carbamate (88 mg, 0.17 mmol) was added 5 mL of a pre-mixed solution of 30% trifluoroacetic acid in dichloromethane. The mixture stirred at ambient temperature for approximately 40 minutes, then solvent was evaporated to residue under a stream of nitrogen. The crude residue was diluted with a 1:1 mixture of water and dimethyl sulfoxide and purified by preparatory HPLC, eluting with 1-20% acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford the trifluoroacetic acid salt of Compound **3** as a clear oil. Yield: 60.3 mg (66%); LCMS m/z 413.3 [M+1]+.

To a solution of N-((2R,3R,4R,5R,6R)-2-((2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)thio)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide trifluoroacetic acid (**3**) (37.6 mg, 0.0714 mmol, 3.3 eq.) and perfluorophenyl 1-azido-15-oxo-17,17-bis((3-oxo-3-(perfluorophenoxy)propoxy)methyl)-3,6,9,12,19-pentaoxa-16-azadocosan-22-oate (**3a**) (23.7 mg, 0.0214 mmol, 1.0 eq.) in 0.5 mL of dimethyl sulfoxide was added triethylamine (20 mL, 0.15 mmol, 6.7 eq.). The mixture stirred under nitrogen atmosphere at ambient temperature for 1 hour, at which time the reaction was diluted further with dimethyl sulfoxide and purified by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford Compound **4** as a clear residue. Yield: 22.7 mg (59%); LCMS m/z 1794.5 [M+1]+, 1791.7 [M-1]-.

To a solution of (**4**) (20.8 mg, 0.0116 mmol) in 3 mL of methanol was added 10% w/w palladium on carbon (14 mg). The solution was degassed under vacuum, then stirred under hydrogen atmosphere using balloon. After 20 minutes, the solution was filtered over Celite and washed with methanol. The filtrate was concentrated to residue and dried further under high vacuum at ambient temperature to afford Compound **5** as a clear film. Yield: 19.1 mg (93%); LCMS m/z 1768.0 [M+1]+.

To a solution of (**5**) (19.0 mg, 0.0108 mmol, 1.0 eq) and perfluorophenyl 4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)benzoate (**Int4**) (4.7 mg, 0.012 mmol, 1.1 eq.) in 0.3 mL of dimethylacetamide, stirring at 0-5C, was added N,N-diisopropylethylamine (8 mL, 0.045 mmol, 4.2 eq.). The mixture continued to stir at 0-5C for approximately 20 minutes until completion, then was diluted with dimethyl sulfoxide and purified by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford Compound **1247** as a white solid. Yield: 11.1 mg (51.7%); LCMS m/z 1967.3 [M+1]+.

Method C (e.g., for the synthesis of Compound **1251**)



To a mixture of (2,3,4,5,6-pentafluorophenyl) 3-[2-(benzyloxycarbonylamino)-3-[3-oxo-3-(2,3,4,5,6-pentafluorophenoxy)propoxy]-2-[[3-oxo-3-(2,3,4,5,6-pentafluorophenoxy)propoxy]methyl]propoxy]propanoate (1.00 eq, 29.0 mg, 0.0299 mmol) and N-[(2R,3R,4R,5R,6R)-2-[5-[2-(2-aminoethoxy)ethoxy]pentyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide;2,2,2-trifluoroacetic acid (**XB47 TFA salt**, 3.10 eq, 45.7 mg, 0.0927 mmol) in DMSO (0.7 mL) was added DIPEA (10.0 eq, 0.052 mL, 0.299 mmol). The mixture

was stirred at room temperature for 1h and was purified by prep. HPLC (2 - 40% MeCN/water with 0.1% TFA) to give **00D** as a white solid (42.2 mg, yield: 91%). LCMS m/z 1552.6 $[M + H]^+$.

To a mixture of **00D** (1.00 eq, 42.2 mg, 0.0272 mmol) in MeOH (4 mL) was added 10% Pd/C (14mg). The mixture was stirred at room temperature under hydrogen for 1h, filtered, concentrated to give **00E** as a white solid. (38.8 mg, yield: 100%) LCMS m/z 1418.8 $[M + H]^+$.

To a mixture of (2,3,4,5,6-pentafluorophenyl) 12-[2-(2,5-dioxopyrrol-1-yl)ethylamino]-12-oxo-dodecanoate (**00C**, 1.50 eq, 10.1 mg, 0.0196 mmol) in DMF (0.7 mL) were added N-[2-[2-[5-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]pentoxy]ethoxy]ethyl]-3-[3-[3-[2-[2-[5-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]pentoxy]ethoxy]ethylamino]-3-oxo-propoxy]-2-[[3-[2-[2-[5-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]pentoxy]ethoxy]ethylamino]-3-oxo-propoxy]methyl]-2-amino-propoxy]midazolidi (**00E**, 1.00 eq, 18.5 mg, 0.0130 mmol) and Diisopropylethylamine (DIPEA) (4.00 eq, 0.0091 mL, 0.0522 mmol). The mixture was stirred at room temperature for 5h and more DIPEA (2.5 mL) was added. The mixture was stirred at room temperature overnight. More DIEA (2 mL) was added, and the mixture was stirred at room temperature for 5h and purified by prep. HPLC (2 - 50%MeCN/water with 0.1%TFA) to give **1251** as a white solid (12.8 mg, yield: 56%). LCMS m/z 1753.7 $[M + H]^+$.

Example 3: Other trivalent ligands with Cys reactive groups

The following section provides details for the preparation of other example trivalent Cys reactive ASGPR target compounds.

Table 10 illustrates various trivalent Cys reactive ASGPR target compounds synthesized using the starting amines indicated in table 16 via the methods indicated or described below.

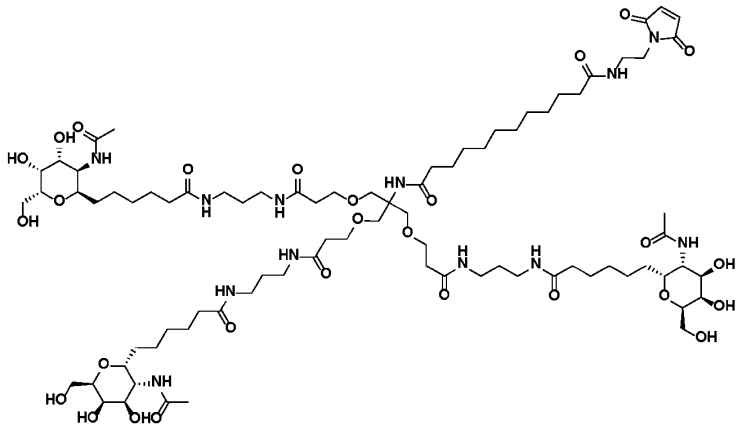
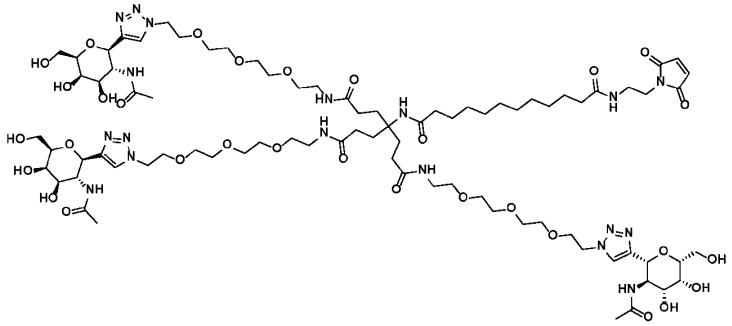
Table 10: Example Trivalent Cys Reactive ASGPR Targeting Compounds		
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1232: 	Method D	1745.0 [M+H]+
1234: 	s/m XB44A, Method E	1870.9 [M + H]$^+$

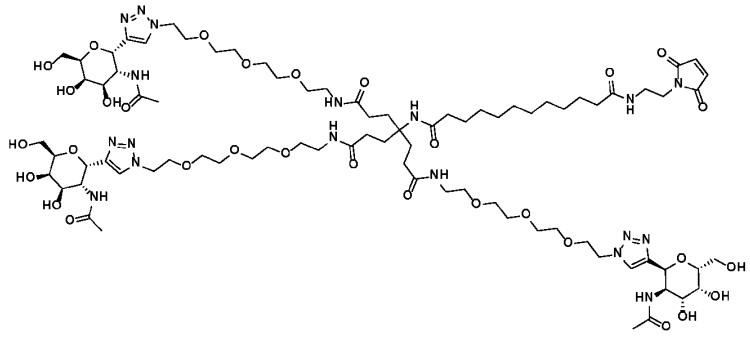
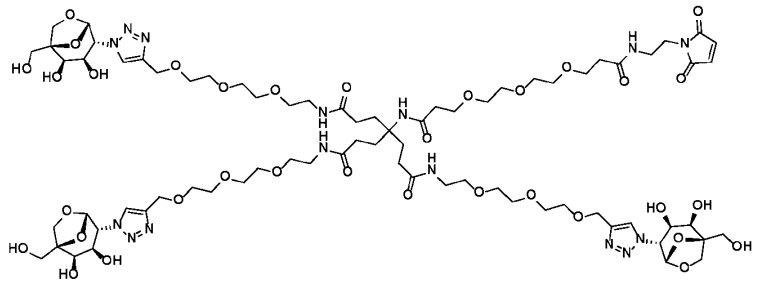
Table 10: Example Trivalent Cys Reactive ASGPR Targeting Compounds		
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1234B: 	s/m **XB44**, adapted from synthesis of 1234 (Method E)	1870.5 [M + H]$^+$
1915: 	Method F	1761.3 [M+H]+

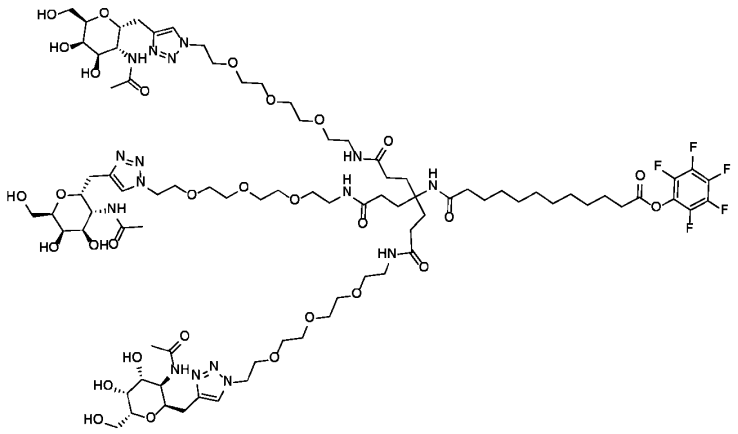
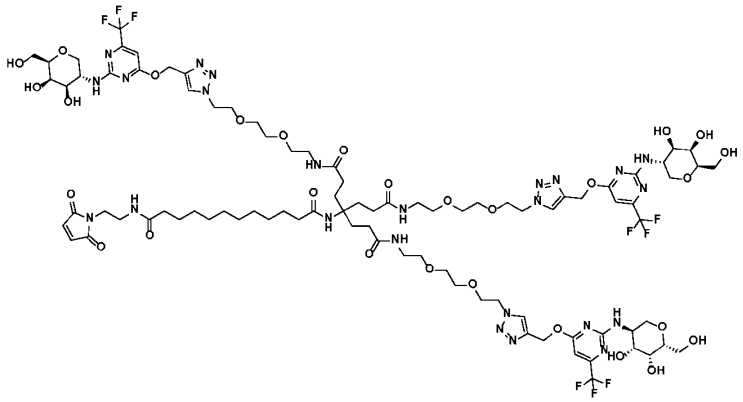
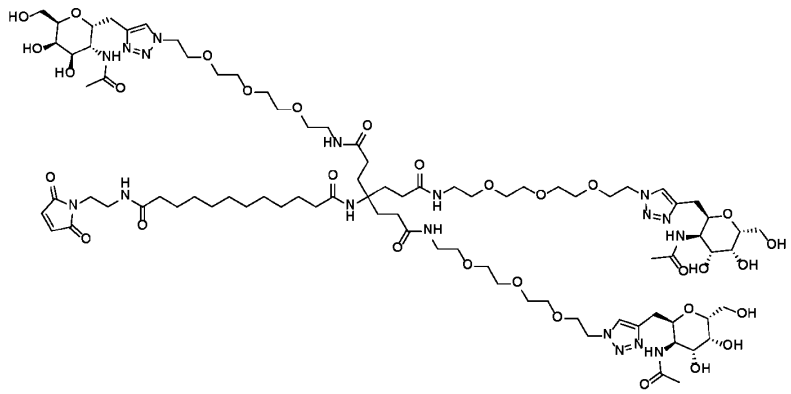
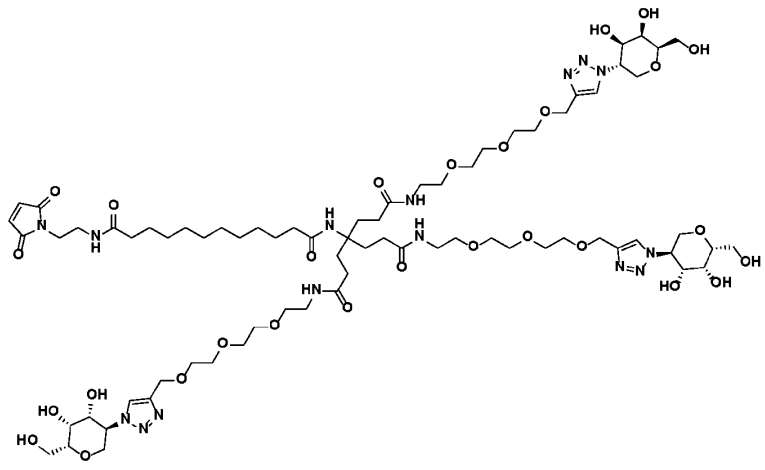
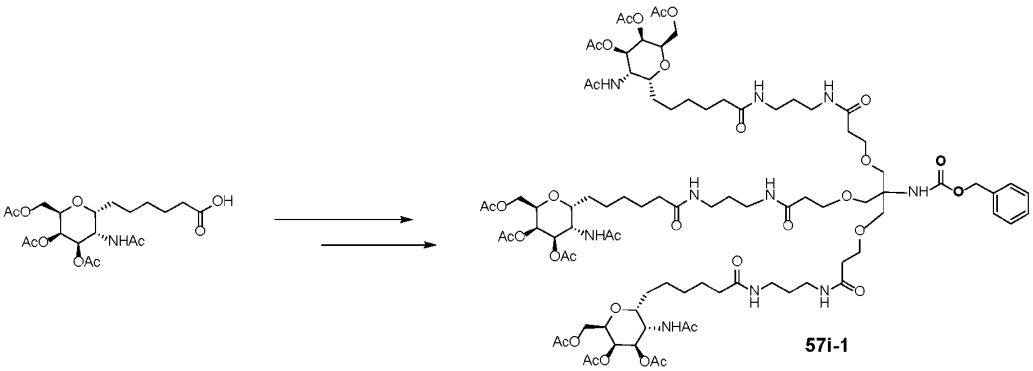
Table 10: Example Trivalent Cys Reactive ASGPR Targeting Compounds		
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1216A: 	s/m **XB4B** and **LC19.1,** adapted from synthesis of **1235**	978.4 [M+2H]++
1905A: 	s/m **XB24** Method G	1070.8 [M+2H]++

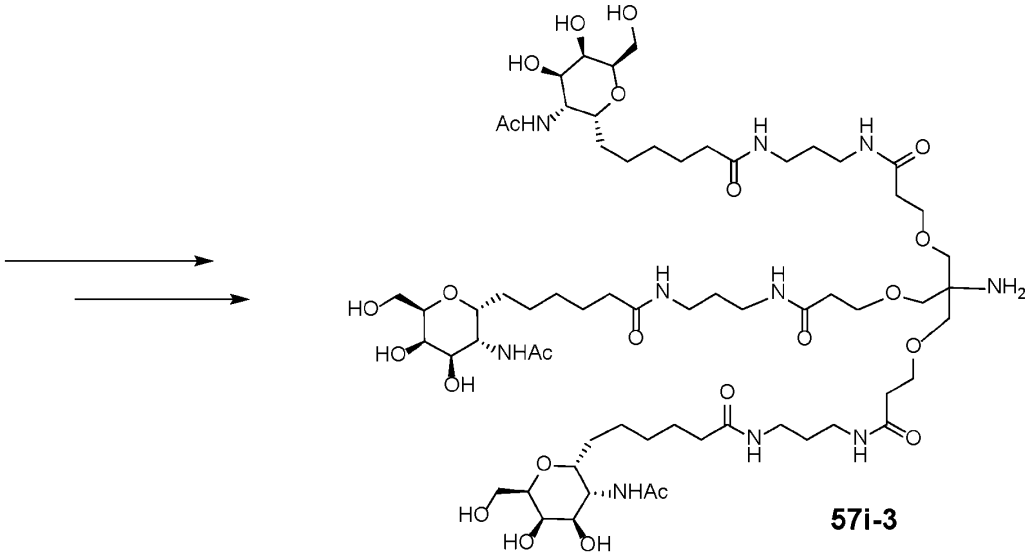
Table 10: Example Trivalent Cys Reactive ASGPR Targeting Compounds		
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1228: 	s/m **XB4B,** adapted from synthesis of **I-168**	1912.6 [M + H]$^+$
1922: 	s/m **XB32,** Method H	1654.8 [M+1]+

Indicated Methods for Table 10 follow:

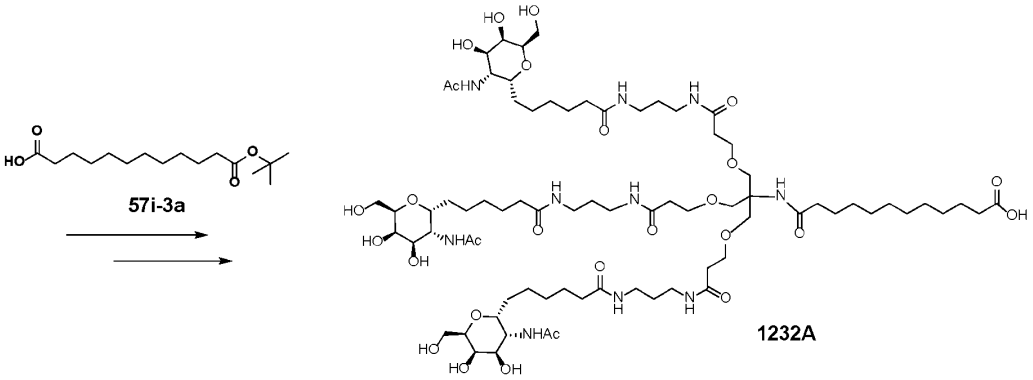
Method D (e.g., for the synthesis of compound 1232):



57i-1

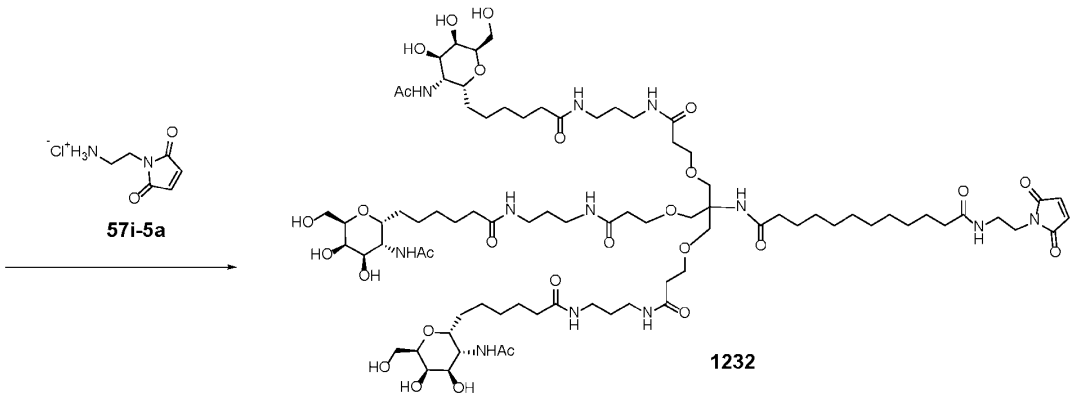


57i-3



57i-3a

1232A



57i-1 was synthesized from 6-((2R,3S,4R,5R,6R)-3-acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)hexanoic acid, which was prepared by literature routes described in Manoharan, et al, WO 2015/006740A2, via chemistry analogous to example 32, intermediates 32-9 described in International Application No. PCT/US2022/037227, filed July 14, 2022.

To a solution of **(57i-1)** (75 mg, 0.039 mmol) in 5 mL of methanol was added 10% w/w palladium on carbon (24 mg). The mixture was degassed under vacuum, then stirred under hydrogen atmosphere via balloon. After 2.5 hrs., the solution was filtered over Celite and washed with methanol. The filtrate was concentrated to residue and dried further under high vacuum at ambient temperature to afford free amine derivative of **57i-1** as a white residue. Yield: 65 mg (93%); LCMS m/z 1787.6 [M+1]+.

To a solution of the free amine derivative of **57i-1** (36 mg, 0.020 mmol) in 1 mL of methanol, stirring under nitrogen atmosphere at 0-5C, was added sodium methoxide (25% w/w in methanol) (85.5 mg, 0.396 mmol, 19 eq) diluted in 0.2 mL of methanol. Following addition, the reaction mixture stirred at ambient temperature for 30 minutes until completion, at which time 0.4 mL of 1N aqueous hydrochloric acid was added slowly to achieve approximate final pH of 1-2. The reaction mixture was diluted further with water and purified by preparatory HPLC, eluting with 1-40% acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford Compound **57i-3** as white solid. Yield: 19.2 mg (68%); LCMS m/z 1409.75 [M+1]+.

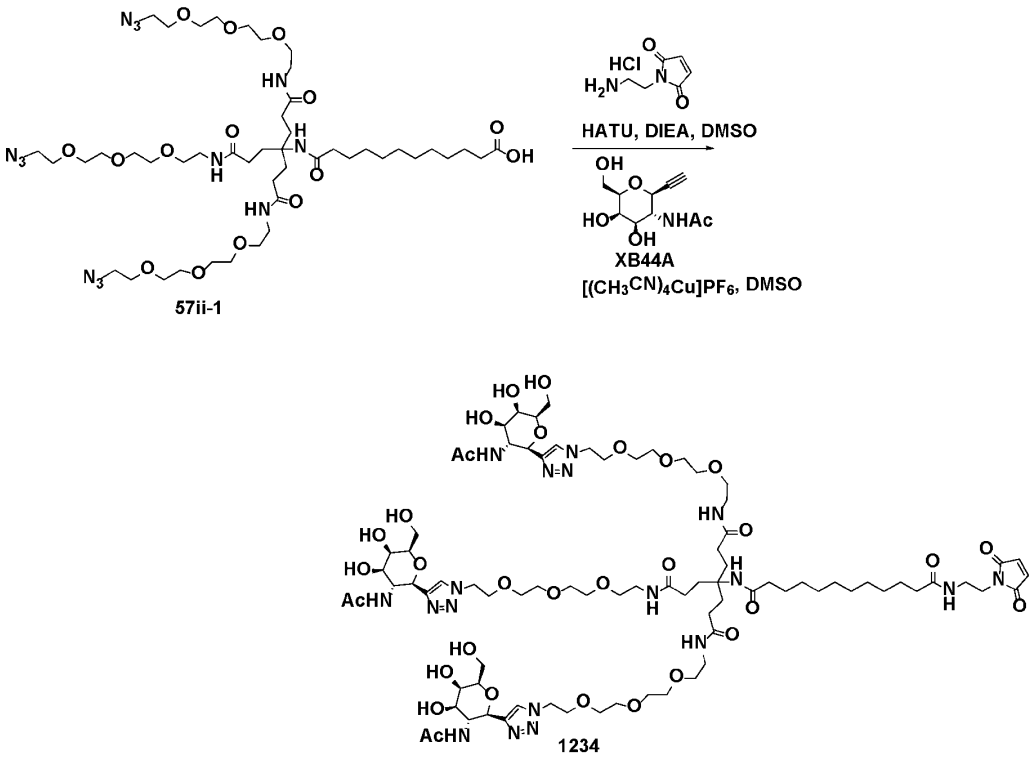
To a solution of 12-(tert-butoxy)-12-oxododecanoic acid **(57i-3a)** (6.8 mg, 0.024 mmol, 1.8 eq.) in 0.1 mL of dimethylformamide was added N,N-diisopropylethylamine (12 mL, 0.069 mmol, 5.1 eq.) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (9.4 mg, 0.025 mmol, 1.8 eq.). The acid activation reaction mixture stirred at ambient temperature for approximately 10 min, followed by addition of **(57i-3)** (19.0 mg, 0.0135 mmol, 1.0 eq.) dissolved in 0.3 mL of dimethylformamide. The reaction continued to stir at ambient temperature until completion, at which time

the mixture was diluted further with dimethyl sulfoxide and purified by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford tert-butyl 1-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-17,17-bis((3-((3-(6-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)hexanamido)propyl)amino)-3-oxopropoxy)methyl)-6,12,19-trioxo-15-oxa-7,11,18-triazatriacontan-30-oate as white solid. Yield: 13.9 mg (61%); LCMS m/z 1678.7 [M+1]+.

To tert-butyl 1-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-17,17-bis((3-((3-(6-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)hexanamido)propyl)amino)-3-oxopropoxy)methyl)-6,12,19-trioxo-15-oxa-7,11,18-triazatriacontan-30-oate (14 mg, 0.0083 mmol) was added 2 mL of a pre-mixed solution of 30% trifluoroacetic acid in dichloromethane. The mixture stirred at ambient temperature for 1 hr., then solvent was evaporated to residue under a stream of nitrogen. The crude residue was diluted with dimethyl sulfoxide and purified by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford Compound **1232A**, as a residue. Yield: 6.38 mg (39%); LCMS m/z 1622.7 [M+1]+.

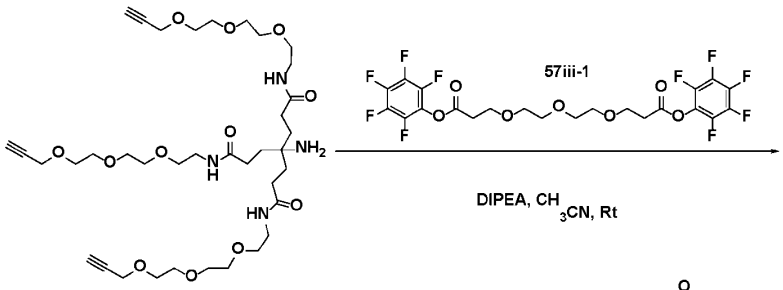
To a solution of 1-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-17,17-bis((3-((3-(6-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)hexanamido)propyl)amino)-3-oxopropoxy)methyl)-6,12,19-trioxo-15-oxa-7,11,18-triazatriacontan-30-oic acid **(1232A)** (6.5 mg, 0.0040 mmol, 1.0 eq.) in 0.3 mL of dimethylformamide was added N,N-diisopropylethylamine (5 mL, 0.029 mmol, 7 eq.) and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (2.3 mg, 0.0060 mmol, 1.5 eq.). The acid activation reaction mixture stirred at ambient temperature for approximately 10 min, followed by addition of 2-maleimidoethylamine hydrochloride **(57i-5a)** (0.92 mg, 0.0052 mmol, 1.3 eq.) in one portion as a solid. The mixture stirred at ambient temperature for approximately 20 minutes until completion, then was diluted with dimethyl sulfoxide and acidified with 2 drops of trifluoroacetic acid. The product was isolated by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford Compound **1232**, as a white solid. Yield: 3.3 mg (65%); LCMS m/z 1745.0 [M+1]+.

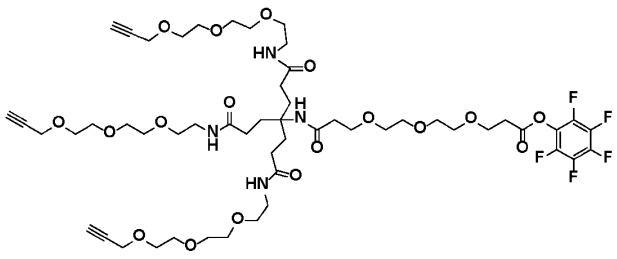
(ii) Method E (e.g., synthesis of Compound 1234):

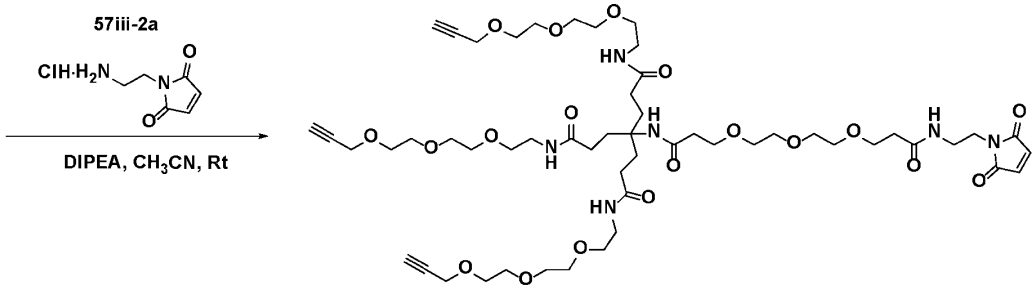


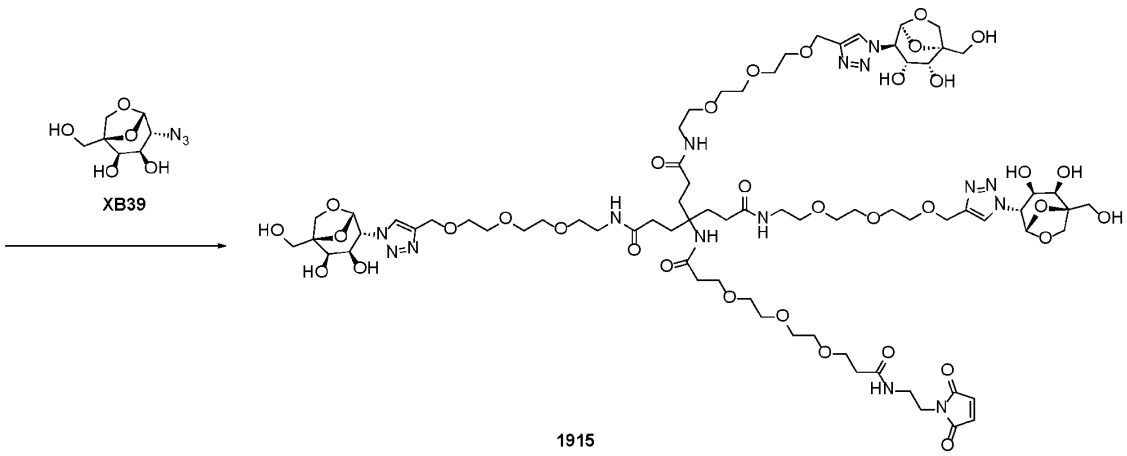
To a mixture of 12-[[4-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethylamino]-1,1-bis[3-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethylamino]-3-oxo-propyl]-4-oxo-butyl]amino]-12-oxo-dodecanoic acid (**57ii-1**, 1.00 eq, 12.9 mg, 0.0122 mmol) in DMSO (0.3 mL) were added Diisopropylethylamine (DIPEA) (4.00 eq, 0.0085 mL, 0.0487 mmol) and HATU (1.10 eq, 5.1 mg, 0.0134 mmol), followed by addition of 2-Maleimidoethylamine hydrochloride (1.00 eq, 2.1 mg, 0.0122 mmol). The mixture was stirred at room temperature for 30 minutes and then N-[(2S,3R,4R,5R,6R)-2-ethynyl-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (**XB44A**, 3.30 eq, 9.2 mg, 0.0402 mmol)was added. The mixture was purged with nitrogen and Tetrakis(acetonitrile)copper(I) hexafluorophosphate (6.00 eq, 27.5 mg, 0.0730 mmol) was added. The mixture was stirred at room temperature for 3h and purified by prep. HPLC (10- 30% MeCN/water with 0.1%TFA) to give **1234** as a white solid (5.5 mg, yield: 24%). LCMS m/z 1870.9 $[M + H]^+$.

(iii) Method F (e.g., for the synthesis of Compound 1915):









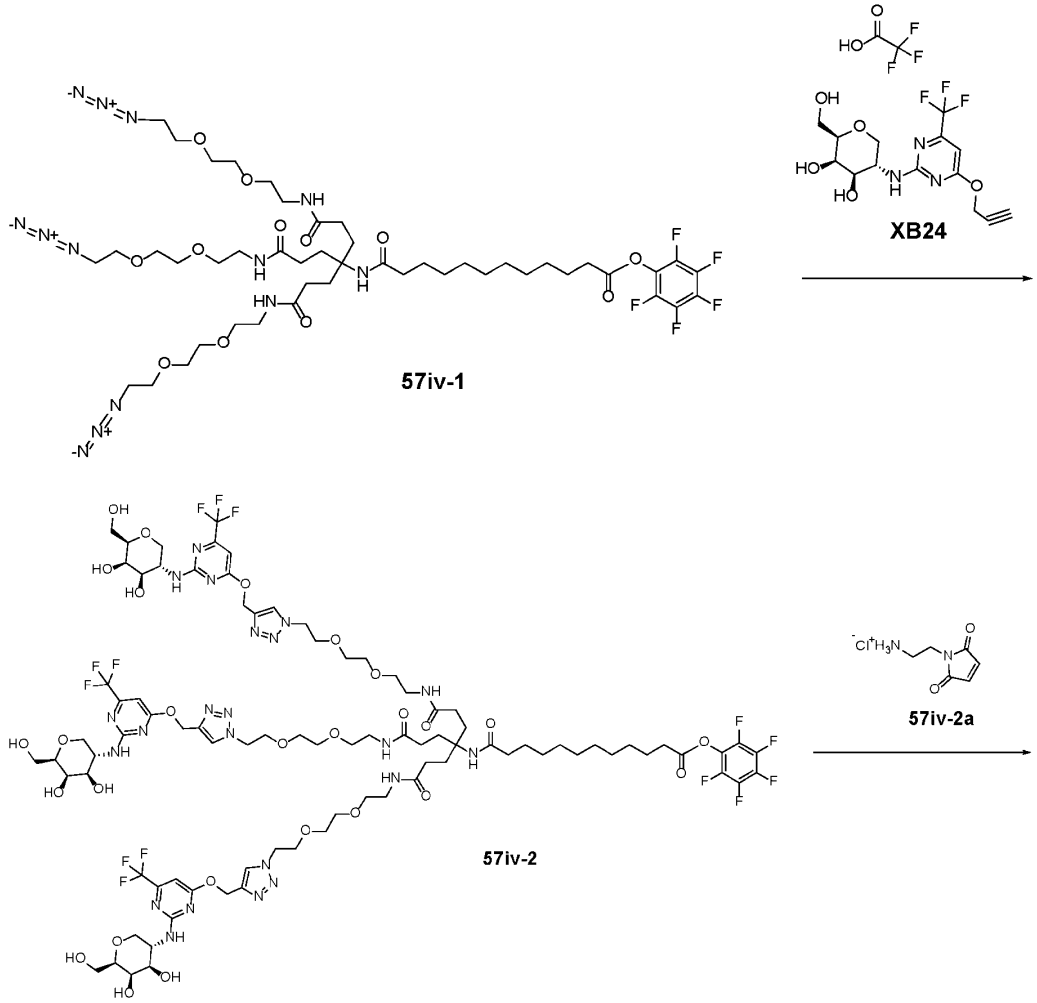
To a stirred solution of bis(perfluorophenyl) 3,3'-((oxybis(ethane-2,1-diyl))bis(oxy))dipropionate (**57iii-1**, 3.0 eq, 1.16 g, 1.99 mmol) and 4-amino-4-(3-oxo-7,10,13-trioxa-4-azahexadec-15-yn-1-yl)-N^1,N^7-bis(2-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethoxy)ethyl)heptanediamide (1.0 eq, 0.5 g, 0.662 mmol) in acetonitrile (5.0 mL), *N,N*-diisopropylethylamine (5.0 eq, 0.578 mL g, 3.31 mmol) was added at 0 °C and reaction mixture was stirred at room temperature for 12 h. After completion (monitored by LCMS), reaction mixture was concentrated under reduced pressure to get crude was purified by prep HPLC (using 30-45% acetonitrile in H_2O with 0.1% TFA) to yield perfluorophenyl 14,19-dioxo-17,17-bis(3-oxo-7,10,13-trioxa-4-azahexadec-15-yn-1-yl)-4,7,10,22,25,28-hexaoxa-13,18-diazahentriacont-1-yn-31-oate (**57iii-2**) as a colorless sticky solid. Yield: 0.450 g, 58.92%; LCMS m/z 1153.35 $[M+1]^+$.

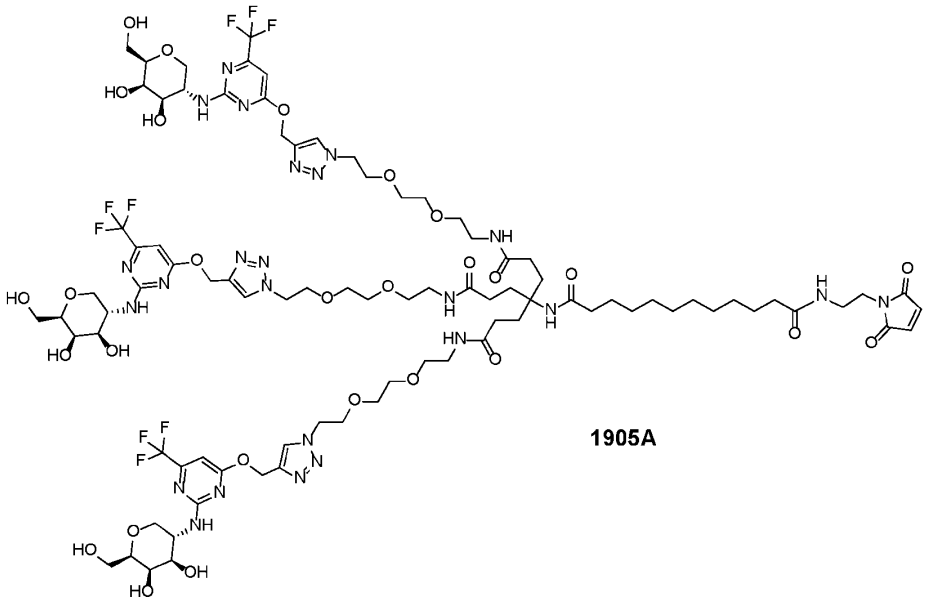
To a stirred solution of perfluorophenyl 14,19-dioxo-17,17-bis(3-oxo-7,10,13-trioxa-4-azahexadec-15-yn-1-yl)-4,7,10,22,25,28-hexaoxa-13,18-diazahentriacont-1-yn-31-oate (**57iii-2,** 1.0 eq., 0.028 g 0.0243 mmol) and 1-(2-aminoethyl)-1H-pyrrole-2,5-dione hydrochloride (**57iii-2a,** 1.5 eq., 0.0063 g 0.0364 mmol) in acetonitrile (2 mL) was added *N,N*-diisopropylethylamine (6.0 eq., 0.00250 mL 0.146 mmol) at 0°C and then stirred at room temperature for 1.5h After completion (monitored by LCMS), reaction mixture was concentrated under reduced pressure to get crude which was purified by prep HPLC (45 55% acetonitrile in water with 0.1 % TFA). Fractions containing the desired product were combined and lyophilized to dryness to afford 4-(1-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-4-oxo-7,10,13-trioxa-3-azahexadecan-16-amido)-4-(3-oxo-7,10,13-trioxa-4-azahexadec-15-yn-1-yl)-N^1,N^7-bis(2-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethoxy)ethyl)heptanediamide (**57iii-2b)** as an off white solid. Yield: 0.008 g, 29.73 %; LCMS m/z 1109.25 $[M+1]^+$; ^{1}H-NMR (400 MHz, DMSO-d_6 with D_2O exchange): δ 6.87 (s, 2H), 4.09 (d, J = 2.0 Hz, 6H), 3.56-3.42 (m, 39H), 3.38 (t, J = 5.6 Hz, 6H), 3.25 (t, J = 2.0 Hz, 2H), 3.18-3.14 (m, 8H), 2.28 (t, J = 6.0 Hz, 2H), 2.20 (t, J = 6.4 Hz, 2H), 2.01-1.97 (m, 6H), 1.77-1.74 (m, 6H).

To a vial was added 8.9 mg (0.0080 mmol) of 4-(1-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-4-oxo-7,10,13-trioxa-3-azahexadecan-16-amido)-4-(3-oxo-7,10,13-trioxa-4-azahexadec-15-yn-1-yl)-N1,N7-bis(2-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethoxy)ethyl)heptanediamide, 6.6 mg (0.029 mmol, 3.8 eq) of (1S,2R,3R,4R,5S)-4-azido-1-(hydroxymethyl)-6,8-dioxabicyclo[3.2.1]octane-2,3-diol (**XB39**, prepared as in Sanhueza, J. Am. Chem. Soc. 2017, 139, 3528−3536) and 0.3 mL DMSO. The mixture stirred at RT until all solids dissolved, then 14.4 mg (0.0386 mmol, 3.9 eq) of tetrakis(acetonitrile)copper(I) hexafluorophosphate was added. The vial was flushed with N2, and reaction stirred at ambient temperature. After 30 min, LCMS indicated consumption of starting tri-alkyne and formation of peak with desired mass [MH+]= 1760.7 (parent, small ionization) and [MH+]= 881.0 (parent+2/2). The reaction mixture was diluted with DMSO and injected onto prep-LC, eluting with MeCN/H2O (0.1%

TFA). Fractions containing desired product were frozen and lyophilized to white solid: **1915,** 8.1 mg (57% yield). LCMS indicated [MH+]= 1761.3 Analysis by ELSD gave 100% area purity.

(iv) Method G (Synthesis of Compound 1905A):





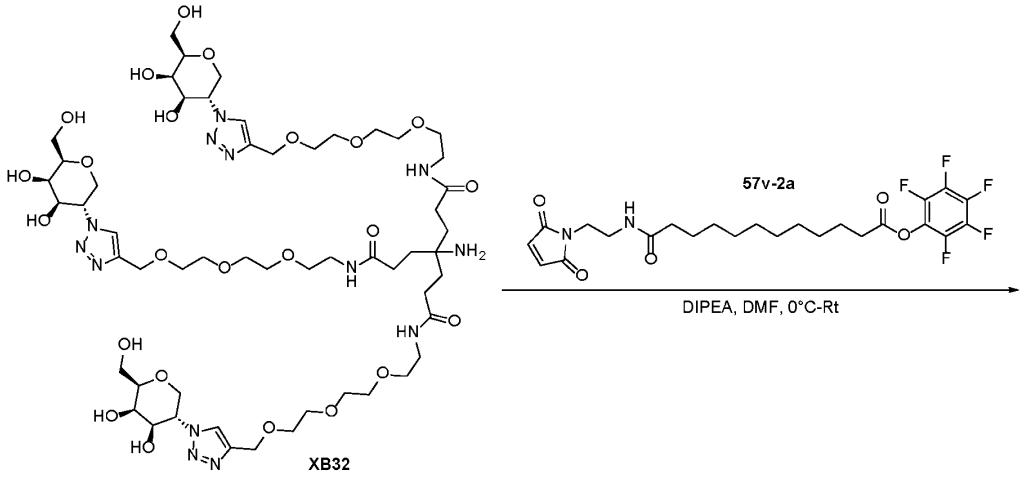
1905A

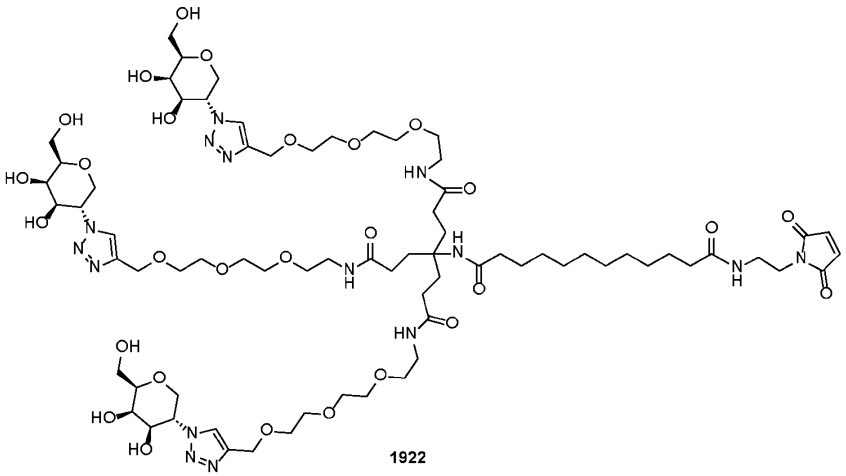
To a solution of perfluorophenyl 1-azido-13,13-bis(3-((2-(2-(2-azidoethoxy)ethoxy)ethyl)amino)-3-oxopropyl)-10,15-dioxo-3,6-dioxa-9,14-diazahexacosan-26-oate (**57iv-1**) (25.2 mg, 0.023 mmol, 1.0 eq.) and (2R,3R,4R,5S)-2-(hydroxymethyl)-5-((4-(prop-2-yn-1-yloxy)-6-(trifluoromethyl)pyrimidin-2-yl)amino)tetrahydro-2H-pyran-3,4-diol trifluoroacetic acid (**XB24 TFA salt**) (36.3 mg, 0.0761 mmol, 3.3 eq.) in 0.5 mL of dimethyl sulfoxide was added tetrakis(acetonitrile)copper(I) hexafluorophosphate(34.5 mg, 0.0926 mmol, 4.0 eq.) in one portion as a solid. The mixture stirred under nitrogen atmosphere at ambient temperature for approximately 50 minutes until consumption of (**57iv-1**). The reaction mixture was diluted further with dimethyl sulfoxide and purified by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford Compound **57iv-2** as white solid. Yield: 35.3 mg (70%); LCMS m/z 1092.2 [M/2+1]+.

To a solution of perfluorophenyl 1-(4-(((2-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)-6-(trifluoromethyl)pyrimidin-4-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)-13,13-bis(3-((2-(2-(2-(4-(((2-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)-6-(trifluoromethyl)pyrimidin-4-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethyl)amino)-3-oxopropyl)-10,15-dioxo-3,6-dioxa-9,14-diazahexacosan-26-oate (**57iv-2**) (10.2 mg, 0.0047 mmol, 1.0 eq.) and 2-maleimidoethylamine hydrochloride (**57iv-2a**) (1.41 mg, 0.0080 mmol, 1.7 eq.) in 0.4 mL of dimethylformamide was added triethylamine (3.5 mL, 0.025 mmol, 5.3 eq.). The mixture stirred under nitrogen atmosphere at ambient temperature for

approximately 30 minutes until consumption of (**57iv-2**). The reaction mixture was diluted further with dimethyl sulfoxide, acidified by addition of 2 drops of trifluoroacetic acid, and purified by preparatory HPLC, eluting with acetonitrile in water with 0.1% trifluoroacetic acid. Fractions containing the desired product were combined and lyophilized to dryness to afford **1905A** as white solid. Yield: 2.6 mg (25%); LCMS m/z 1070.8 [M/2+1]+.

(v) Method H (synthesis of 1922):





To a solution of 4-amino-4-(1-(1-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)-1H-1,2,3-triazol-4-yl)-12-oxo-2,5,8-trioxa-11-azatetradecan-14-yl)-N1,N7-bis(2-(2-((1-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)-1H-1,2,3-triazol-4-

yl)methoxy)ethoxy)ethoxy)ethyl)heptanediamide (**XB32**, 0.210 g, 1.0 eq, 0.159 mmol) and perfluorophenyl 12-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-12-oxododecanoate (**57v-2a,** 0.124 g, 1.5 eq, 0.238 mmol) in dimethyl sulfoxide (3.0 mL), *N,N*-diisopropylethylamine (5.0 eq, 0.138 mL, 794 mmol) was added and reaction mixture was stirred at room temperature for 16h. After completion (monitored by LCMS), reaction mixture was concentrated to afford crude which was purified by prep HPLC (35-65 % acetonitrile in water with 0.05% TFA). Fractions containing the desired compound were combined and lyophilized to afford N1-(1,29-bis(1-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)-1H-1,2,3-triazol-4-yl)-15-(1-(1-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)-1H-1,2,3-triazol-4-yl)-12-oxo-2,5,8-trioxa-11-azatetradecan-14-yl)-12,18-dioxo-2,5,8,22,25,28-hexaoxa-11,19-diazanonacosan-15-yl)-N12-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)dodecanediamide (**1922**) as white solid. Yield: 0.052 g, 19.76%. LCMS m/z 1654.80 $[M-1]^-$; ^{1}H-NMR (400 MHz, DMSO-d_6 with D_2O): δ 8.06 (s, 3H), 6.87 (s, 2H), 4.66-4.62 (m, 3H), 4.49 (s, 6H), 4.00-3.93 (m, 7H), 3.63 (t, J = 11.6 Hz, 5H), 3.53-3.47 (m, 35H), 3.42 (t, J = 6.4 Hz, 3H), 3.37 (t, J = 6.0 Hz, 6H), 3.18-3.13 (m, 9H), 3.15-3.13 (m, 6H), 2.01-1.91 (m, 11H), 1.78-1.76 (m, 6H), 1.41-1.37 (m, 5H), 1.21-1.11 (m, 14H).

Table 11 illustrates various divalent Cys or Lys reactive ASGPR target compounds synthesized via the methods indicated or described herein.

Table 11: Example Divalent Cys or Lys Reactive ASGPR Targeting Compounds

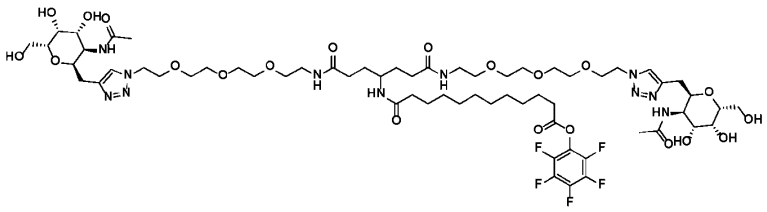
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1235: 	s/m **XB4B**	1440.3 $[M+H]^+$

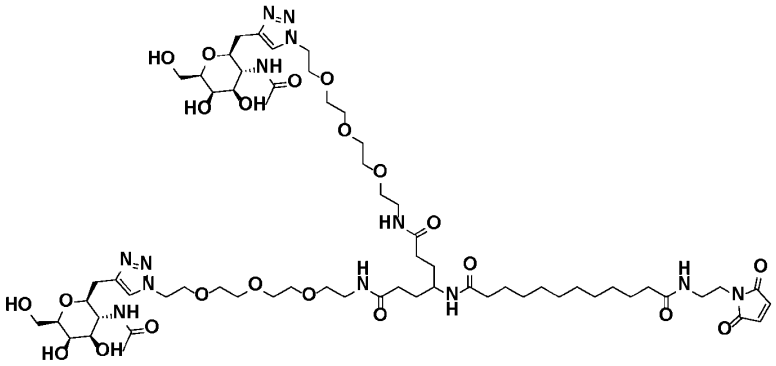
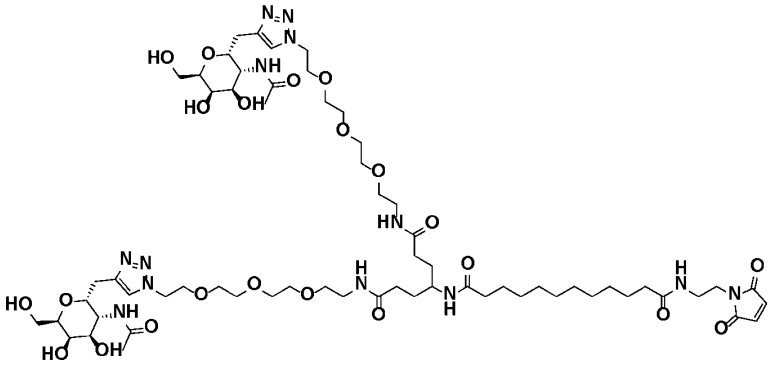
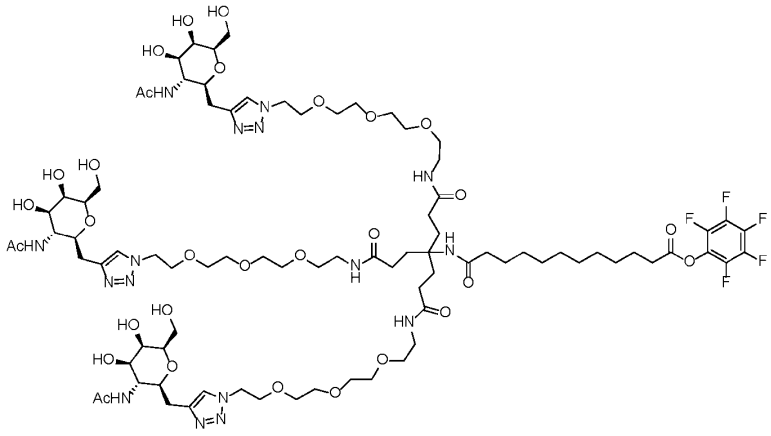
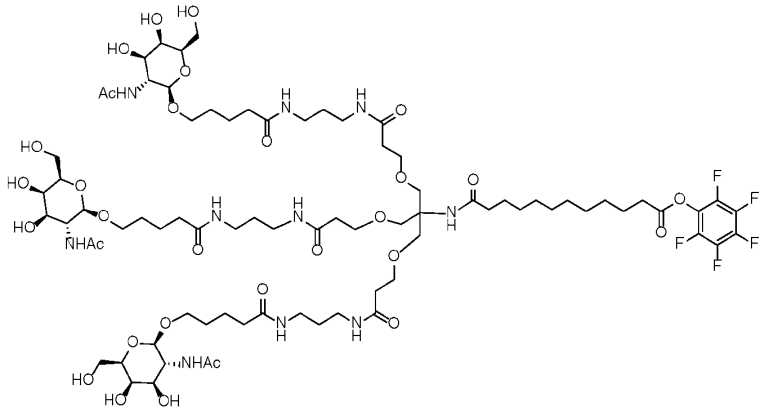
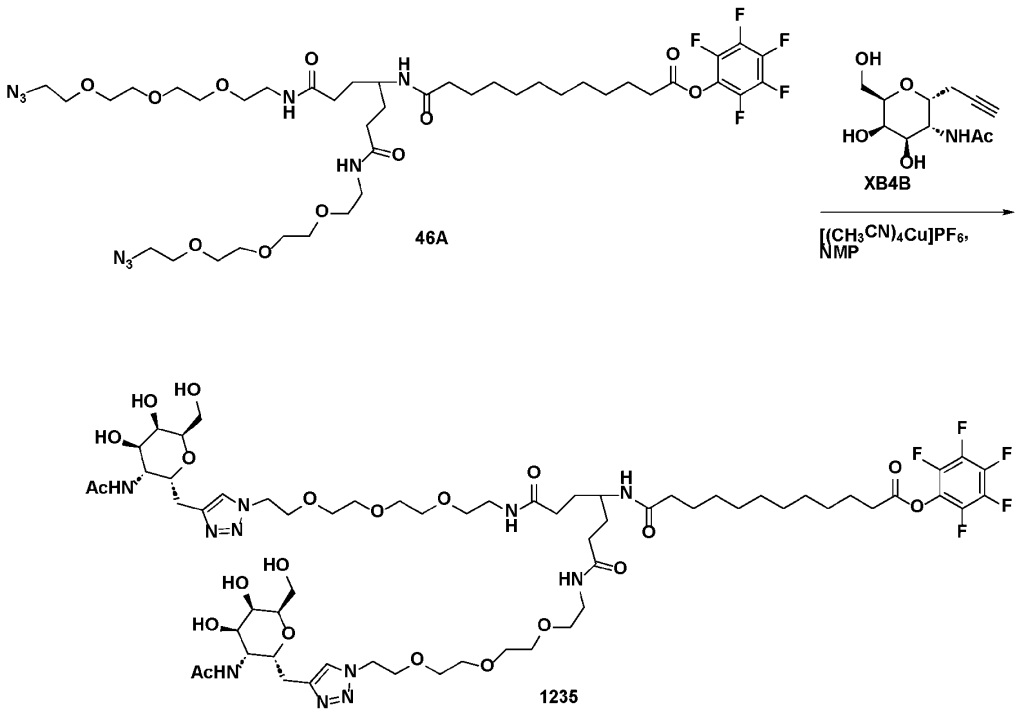
Table 11: Example Divalent Cys or Lys Reactive ASGPR Targeting Compounds		
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1217: 	s/m **XB4** Adapted from the synthesis of compound **I-168**	1396.2 [M+H]+
1217A: 	s/m **XB4B** Adapted from the synthesis of **1217**	

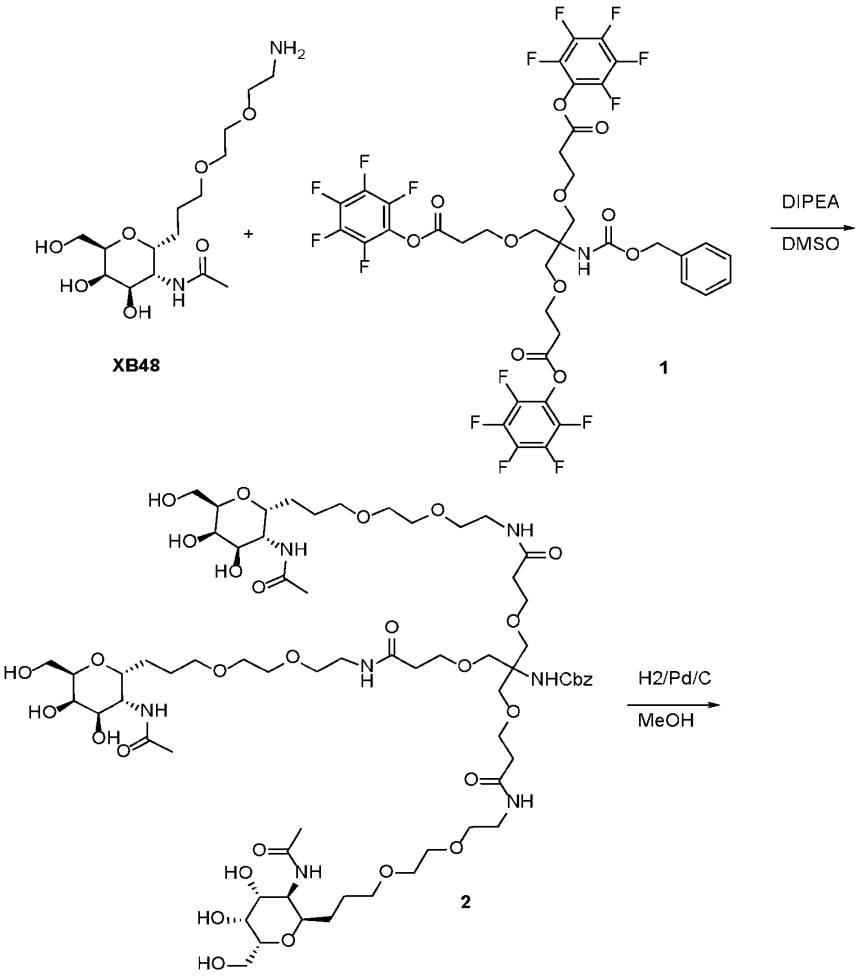
Table 11: Example Divalent Cys or Lys Reactive ASGPR Targeting Compounds		
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1216: 	WO2023288033	
1209: 	WO2023288033	

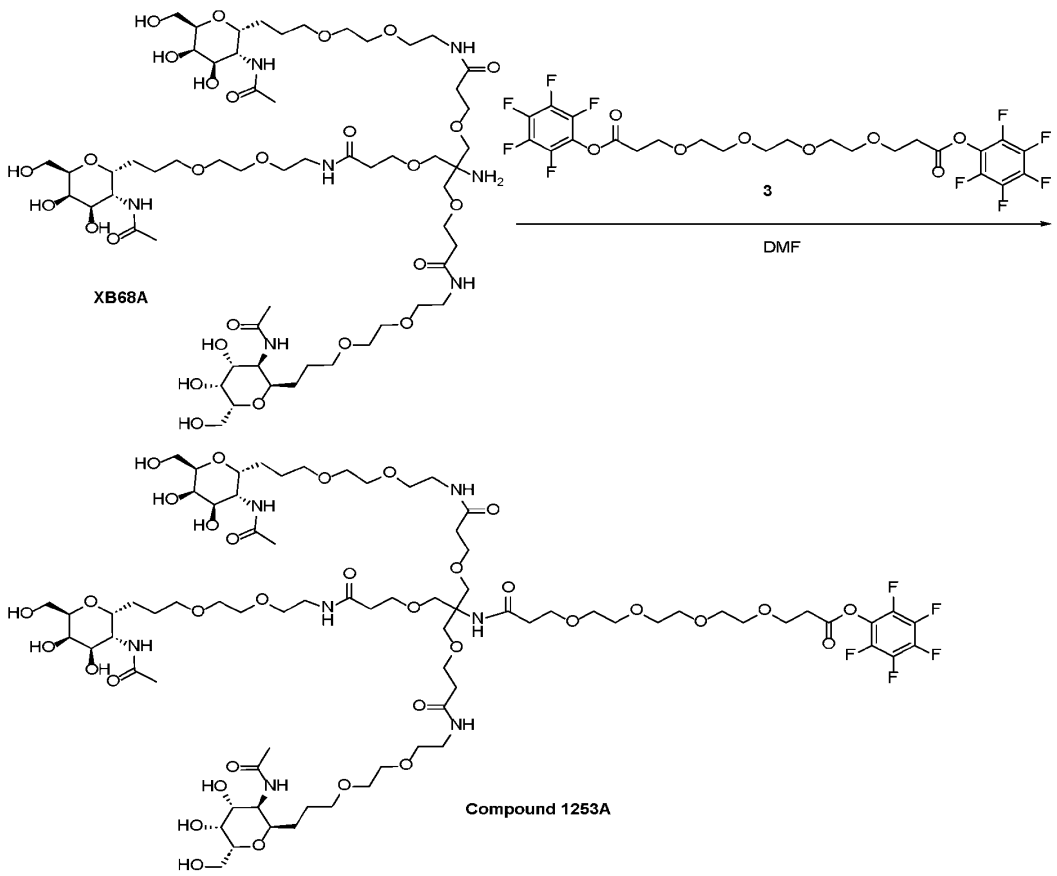
Synthesis of Compound **1235**:



To a mixture of (2,3,4,5,6-pentafluorophenyl) 12-[[4-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethylamino]-1-[3-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethylamino]-3-oxo-propyl]-4-oxo-butyl]amino]-12-oxo-dodecanoate (**46A**, 1.00 eq, 15.6 mg, 0.0164 mmol) and N-[(2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-prop-2-ynyl-tetrahydropyran-3-yl]acetamide (**XB4B**, 2.30 eq, 9.1 mg, 0.0376 mmol) in NMP (0.25 mL) was added Tetrakis(acetonitrile)copper(I) hexafluorophosphate (4.00 eq, 24.6 mg, 0.0654 mmol). The mixture was stirred at room temperature for 1.5h. The mixture was purified by prep. HPLC (20 - 60% MeCN/water with 0.1% TFA) to give **1235** as a white solid (12.7 mg, Yield: 54%). LCMS m/z 1440.3 $[M + H]^+$.

Synthesis of Compound 1253A:





Synthesis of benzyl (1,31-bis((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-16-(15-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-5-oxo-2,9,12-trioxa-6-azapentadecyl)-11,21-dioxo-4,7,14,18,25,28-hexaoxa-10,22-diazahentriacontan-16-yl)carbamate (**2**)

A mixture of N-((2R,3R,4R,5R,6R)-2-(3-(2-(2-aminoethoxy)ethoxy)propyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (**XB48**, 3.24 eq, 1.72 g, 4.91 mmol) and bis(perfluorophenyl) 3,3'-((2-(((benzyloxy)carbonyl)amino)-2-((3-oxo-3-(perfluorophenoxy)propoxy)methyl)propane-1,3-diyl)bis(oxy))dipropionate (1.00 eq, 1.47 g, 1.52 mmol) was treated with diisopropylethylamine (**1**, 3.00 eq, 862 mL, 4.95 mmol) then dissolved in DMSO (6.89 mL) with sonication. After 90 minutes the reaction was purified by reversed-phase HPLC (5% then 15-50% acctonitrilc in watcr w/0.1% FA) to givc **2**. Yicld: 2.21 g, 91%. LCMS 1468.6 [M+H].

Synthesis of N-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethyl]-3-[3-[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-

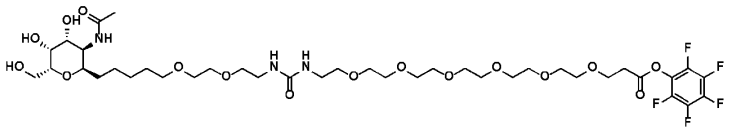
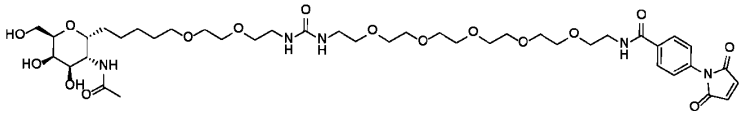
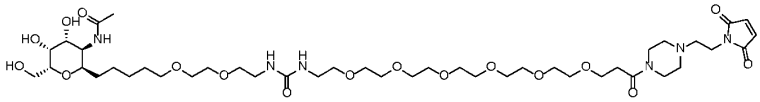
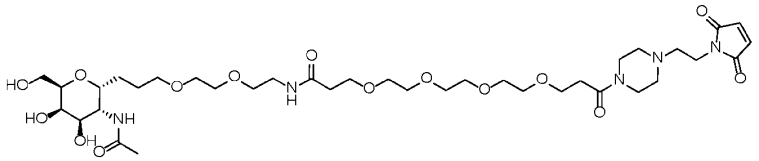
acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]-2-[[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]methyl]-2-amino-propoxy]propenamide (**XB68A**)

A solution of **2** (1.00 eq, 400 mg, 0.272 mmol) in methanol (20 mL) was purged with nitrogen then treated with 10% Pd/C (0.400 eq, 232 mg, 0.109 mmol) before being evacuated then back-filled with hydrogen via balloon. After 2h, the reaction was treated with 500 mg celite then filtered over a pad of celite. The filter cake was rinsed with methanol and the filtrate was concentrated under reduced pressure. The residue was dissolved in water with minimal DMSO then purified by reversed-phase HPLC (5-50% acetonitrile in water w/ 0.2 mM NH_4OH) to give **XB68A**. Yield: 315 mg, 86.7%. LCMS 1335.6 [M+H].

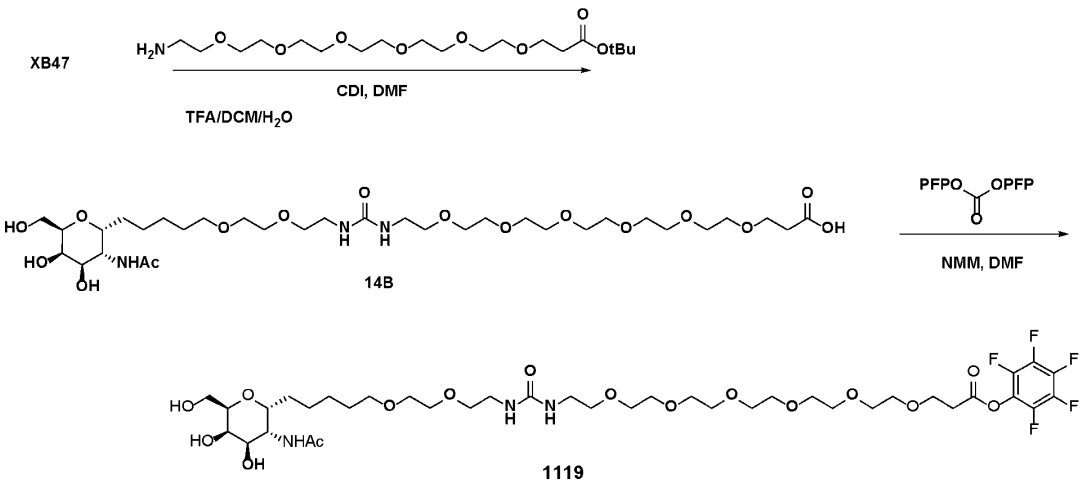
Synthesis of perfluorophenyl 33-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-18,18-bis(15-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-5-oxo-2,9,12-trioxa-6-azapentadecyl)-16,23-dioxo-4,7,10,13,20,27,30-heptaoxa-17,24-diazatritriacontanoate (**Compound 1253A**)

A solution of **XB68A** (1.00 eq, 1.10 g, 0.824 mmol) in DMF (13.7 mL) was added to a solution of bis(perfluorophenyl) 4,7,10,13-tetraoxahexadecanedioate (**3**, 3.00 eq, 1.55g, 2.47 mmol) in DMF (5.6 mL) drop-wise via syringe over 16 minutes. Next, DMF (2 mL) was added to the amine flask for rinsing then this solution was added to the reaction dropwise via syringe. The reaction was stirred at room temperature overnight. After 26h, the reaction was purified directly by reversed-phase HPLC (5-15-40% acetonitrile in water w/0.1% TFA) to give solids that were not easily manipulated. The residue was treated with acetonitrile to remove water via azeotropic distillation. The residue was then dissolved in MeOH (1 volume) then diluted with DCM (95 volumes) then concentrated under high vacuum for 18h to give **Compound 1253A** as a powder. Yield: 1050 mg, 71%. LCMS 1777.58 [M+H].

Table 12 illustrates various monovalent Cys or Lys reactive ASGPR target compounds synthesized via the methods indicated or described below.

Table 12: Example Monovalent Cys or Lys Reactive ASGPR Targeting Compounds		
Compound number and structure	**Starting material (s/m) and synthesis method**	**LCMS m/z observed**
1119: 	s/m **XB47**	924.4 [M + H]$^+$
1120: 	s/m **XB47**	884.3 [M + H]+
1122: 	s/m **XB47** Adapted from the synthesis of **1120**	949.8 [M+H]+
1123: 	s/m **XB48** Method A	818.8 [M+H]+

Synthesis of Compound **1119**:



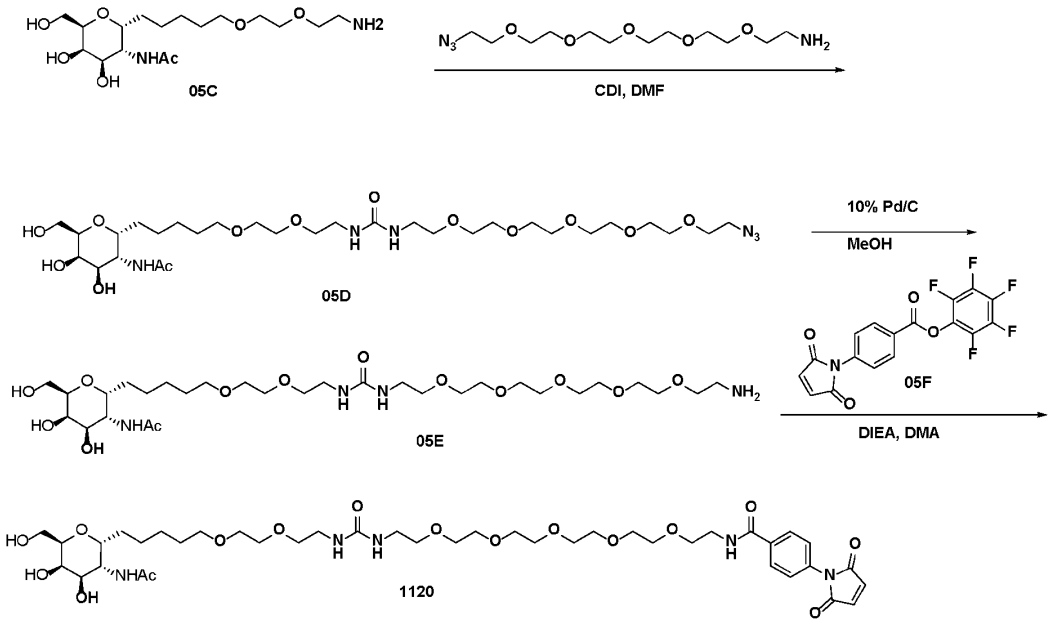
To a mixture of carbonyldiimidazole (1.30 eq, 18.1 mg, 0.112 mmol) in DMF (0.4 mL) was added a solution of Amino-PEG6-t-butyl ester (1.30 eq, 45.7 mg, 0.112 mmol) in DMF (0.4 mL). The mixture was stirred at room temperature for 1h, then N-[(2R,3R,4R,5R,6R)-2-[5-[2-(2-aminoethoxy)ethoxy]pentyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (**XB47**, 1.00 eq, 32.5 mg, 0.0859 mmol) was added and more DMF (0.6 mL) was added. The mixture was stirred at room temperature for 5h and purified by prep. HPLC (9 - 50% MeCN/water with 0.1%FA) to give tert-butyl 3-[2-[2-[2-[2-[2-[2-[2-[5-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]pentoxy]ethoxy]ethylcarbamoylamino]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoate as a white solid (55 mg, yield: 79%). LCMS m/z 814.3 $[M + H]^+$.

To a mixture of tert-butyl 3-[2-[2-[2-[2-[2-[2-[2-[5-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]pentoxy]ethoxy]ethylcarbamoylamino]ethoxy]ethoxy]ethoxy]ethoxy]ethoxy]propanoate (1.00 eq, 55.0 mg, 0.0676 mmol) in DCM (1 mL) was added a solution of TFA (1 mL) and water (100 uL). The mixture was stirred at rt for 30 minutes, concentrated, co-evaporated with water (2x), and lyophilized to give **14B (1121)** as clear syrup (54.6 mg, yield: 107%). LCMS: 758.4 $[M + H]^+$.

To a mixture of **1121** (1.00 eq, 25.5 mg, 0.0336 mmol) in DMF (0.6 mL) at 0 °C was added Bis(pentafluorophenyl) carbonate (1.00 eq, 13.3 mg, 0.0336 mmol), followed by addition of a solution of 4-Methylmorpholine (1.00 eq, 0.0037 mL, 0.0336 mmol) in DMF (30 uL). The mixture was

stirred at 0 °C for 30 minutes and purified by prep. HPLC (10 - 70% MeCN/water with 0.1% TFA) to give **1119** as a white solid (17.3 mg, yield: 56%). LCMS m/z 924.4 $[M+H]^+$.

Synthesis of Compound **1120**:



To a mixture of Carbonyldiimidazole (1.40 eq, 10.8 mg, 0.0666 mmol) in DMF (0.2 mL) was added a solution of 2-[2-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethanamine (1.40 eq, 20.4 mg, 0.0666 mmol) in DMF (0.3 mL). The mixture was stirred at room temperature for 2h, then N-[(2R,3R,4R,5R,6R)-2-[5-[2-(2-aminoethoxy)ethoxy]pentyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (**05C, XB47**

, 1.00 eq, 18.0 mg, 0.0476 mmol) was added. The mixture was stirred at room temperature for 5h and was purified by prep. HPLC (9 - 50% MeCN/water with 0.1% TFA) to give **05D** as a white solid (26.8 mg, yield: 79%). LCMS m/z 711.3 $[M + H]^+$.

To a mixture of N-[(2R,3R,4R,5R,6R)-2-[5-[2-[2-[2-[2-[2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethylcarbamoylamino]ethoxy]ethoxy]pentyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (**05D**, 1.00 eq, 30.6 mg, 0.0430 mmol) in MeOH (4 mL) was added 10% Pd/C (13 mg). The mixture was stirred at room temperature under hydrogen for 30

minutes, filtered, concentrated, and purified by prep. HPLC (2- 50% MeCN/20mM NH_4OH aqueous solution) to give **05E, XB70** as a white solid (14.9 mg, yield: 66%). LCMS m/z 685.4 $[M + H]^+$.

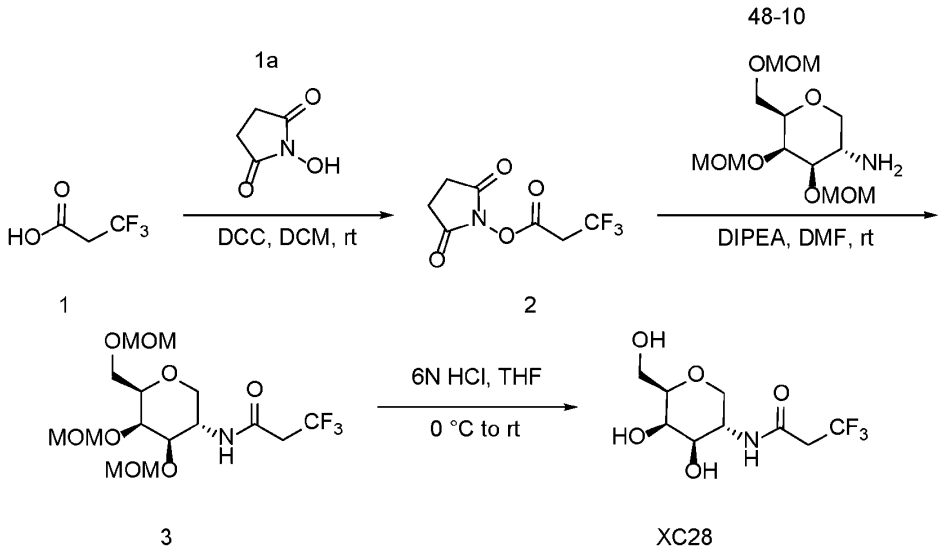
To a mixture of 2,3,4,5,6-Pentafluorophenol (1.20 eq, 300 mg, 1.63 mmol) and 4-Maleimidobenzoic acid (1.00 eq, 295 mg, 1.36 mmol) in DCM (6 mL) was added 1,3-diisopropylcarbodiimide (1.50 eq, 0.32 mL, 2.04 mmol). The mixture was stirred at room temperature for 2h and filtered. The filtrate was concentrated and purified by column (0- 25% EtOAc/hexane) to give **05F** as a white solid (449 mg, yield: 86%). 1H NMR (400 MHz, CDCl3) δ 8.37 - 8.28 (m, 2H), 7.72 - 7.62 (m, 2H), 6.95 (s, 2H)

To a mixture of (2,3,4,5,6-pentafluorophenyl) 4-(2,5-dioxopyrrol-1-yl)benzoate (**05F**, 1.10 eq, 11.9 mg, 0.0312 mmol) in DMA (0.1 mL) at 0°C was added a solution of N-[(2R,3R,4R,5R,6R)-2-[5-[2-[2-[2-[2-[2-[2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]ethoxy]ethoxy]ethylcarbamoylamino]ethoxy]ethoxy]pentyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (**05E**, 1.00 eq, 19.4 mg, 0.0283 mmol) in DMA (0.3 mL) dropwise. The mixture was stirred at 0 °C for 10 minutes. DIPEA (1.00 eq, 0.0049 mL, 0.0283 mmol) was added. The mixture was stirred at 0 °C for 20 minutes. The mixture was purified by prep. HPLC (9 - 40 % MeCN/water with 0.1%formic acid) to give **1120** as a white solid (15.5 mg, yield: 62%). LCMS: 884.3. $[M + H]^+$.

Example 4: Synthesis of 2R and 6R modification Compounds

The following section provides details for the preparation of example building blocks with 2R and 6R modifications.

Synthesis of compound **XC28**:



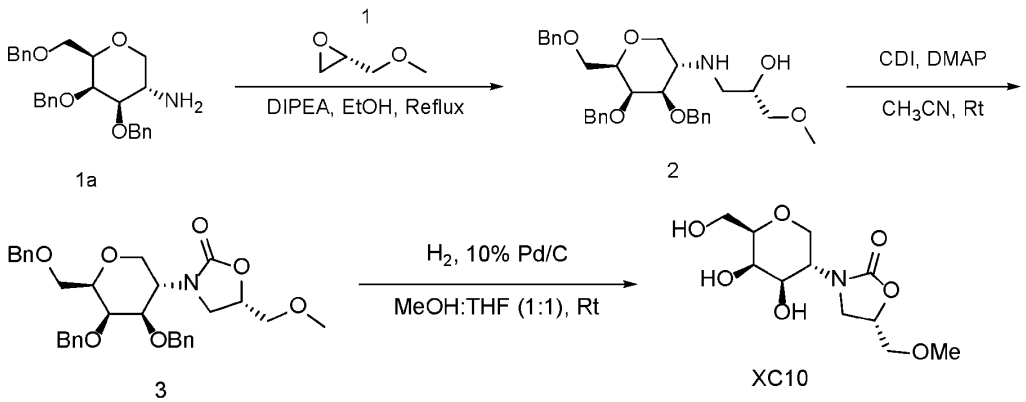
1 2 3 XC28

To a solution of 3,3,3-trifluoropropanoic acid (**1**, 1.0 eq, 0.100 g, 0.781 mmol) in dichloromethane (2 mL), N-hydroxysuccinimide (**1a**, 1.5 eq, 0.134 g, 1.17 mmol) and N,N'-dicyclohexylcarbodiimide (2.0 eq, 0.321 mL, 1.56 mmol) were added and reaction mixture was stirred at room temperature for 16 h. After completion, reaction mixture was concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-5 % methanol in dichloromethane to afford 2,5-dioxopyrrolidin-1-yl 3,3,3-trifluoropropanoate (**2**) as a colourless viscous liquid. Yield: 0.050 g, 28.57 %; LCMS m/z No ionization; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.54-3.47 (m, 2H), 2.87 (s, 4H).

To a solution of (3S,4R,5R,6R)-4,5-bis(methoxymethoxy)-6-((methoxymethoxy)methyl)tetrahydro-2H-pyran-3-amine (**48-10**, 1.0 eq, 0.070 g, 0.237 mmol) in N,N-dimethylformamide (1 mL), 2,5-dioxopyrrolidin-1-yl 3,3,3-trifluoropropanoate (**2**, 1.5 eq, 0.080 g, 0.356 mmol) and N,N-diisopropylethylamine (3.0 eq, 0.12 mL, 0.711 mmol) were added and reaction mixture was stirred at room temperature for 16 h. After completion, water was added to reaction mixture and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate, filtered and concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-20 % ethyl acetate in hexane to afford N-((3S,4R,5R,6R)-4,5-bis(methoxymethoxy)-6-((methoxymethoxy)methyl)tetrahydro-2H-pyran-3-yl)-3,3,3-trifluoropropanamide (**3**) as a light yellow viscous liquid. Yield: 0.030 g, 31.22 %; LCMS m/z 423.20 $[M+18]^+$.

To a solution of N-((3S,4R,5R,6R)-4,5-bis(methoxymethoxy)-6-((methoxymethoxy)methyl)tetrahydro-2H-pyran-3-yl)-3,3,3-trifluoropropanamide (**3,** 1.0 eq, 0.035 g, 0.086 mmol) in tetrahydrofuran (0.5 mL) was cooled at 0 °C, 6N hydrochloric acid (0.5 mL) was added and reaction mixture was stirred at room temperature for 16 h. After completion, reaction mixture was concentrated to get crude which was purified by prep HPLC (25-33 % acetonitrile in water with 0.1 % trifluoroacetic acid). Fractions containing the desired product were combined and lyophilized to dryness to afford N-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)-3,3,3-trifluoropropanamide (Cpd. No. **XC28**) as a cream solid. Yield: 0.008 g, 33.91 %; LCMS m/z 274.10 $[M+1]^+$; ^{1}H NMR (400 MHz, MeOD) δ 4.18-4.12 (m, 1H), 3.98-3.93 (m, 1H), 3.86 (d, *J* = 3.2 Hz, 1H), 3.75-3.70 (m, 1H), 3.67-3.63 (m, 1H), 3.52-3.49 (m, 1H), 3.41-3.38 (m, 1H), 3.21-3.13 (m, 2H), 3.08 (t, *J* = 10.8 Hz, 1H).

Synthesis of Compound **XC10**:



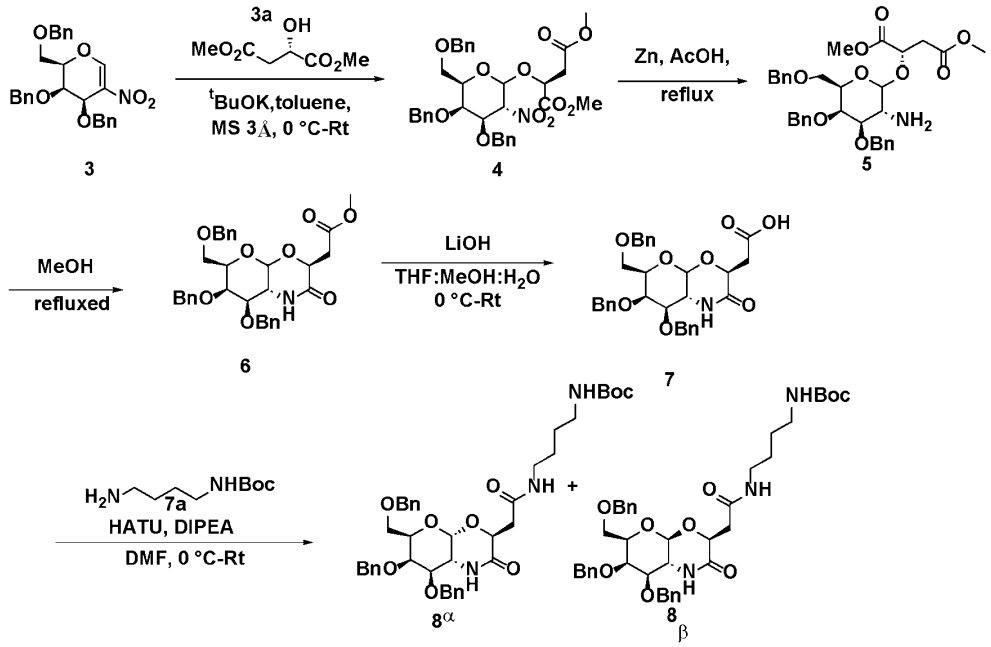
To a stirred solution of (3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-amine (**1a,** 1.0 eq.,0.50 g.,4.11 mmol.) and (S)-2-(methoxymethyl)oxirane (**1,** 1.0 eq., 0.0914 g, 1.04 mmol.) in ethanol (5.0 mL) was added N,N-Diisopropylethylamine (2.0 eq,.0.403 mL, 2.31 mmol.), and reaction mixture was refluxed for 12 h. After completion reaction (monitored by LCMS.), ethanol was evaporated under reduce pressure to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-10 % methanol in dichloromethane to afford (S)-1-(((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)amino)-3-methoxypropan-2-ol (**2**) as an off white solid. Yield: 0.47 g, 78.12 %; LCMS m/z 522.15 $[M+1]^+$.

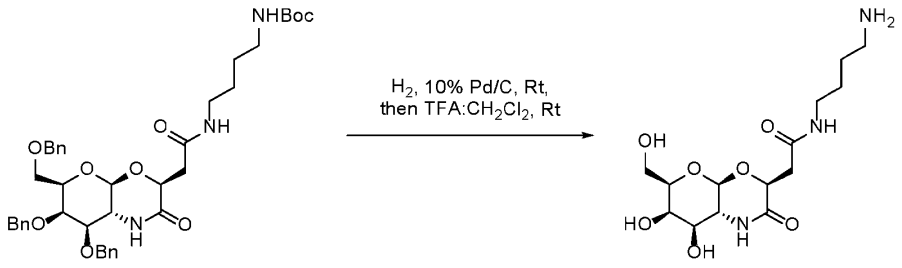
To a stirred solution of (S)-1-(((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)amino)-3-methoxypropan-2-ol (**2,** 1.0 eq., 0.37 g., 0.709 mmol) in acetonitrile (4.0 mL), 1,1'-carbonyldiimidazole (CDI) (1.5 eq., 0.173 g., 1.06 mmol) and *N,N*-

dimethylpyridin-4-amine (, 0.1 eq., 8.67 mg 0.070 mmol) were added and reaction mixture was stirred at room temperature for 16h. After completion (monitored by LCMS), reaction mixture was concentrated under reduce pressure to get crude which was purified by flash column chromatography on silica gel using 0-50% ethyl acetate in hexane to afford a (S)-3-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)-5-(methoxymethyl)midazolidi-2-one (**3**) as a colorless liquid. Yield: 0.2 g; 51.49 %, LCMS m/z 548.0 $[M+1]^+$.

To a solution of (S)-3-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)-5-(methoxymethyl)midazolidi-2-one (**3,** 1.0 eq., 0.2 g, 0.365 mmol) in methanol (2.0 mL) and tetrahydrofuran (2.0 mL) was added 10% palladium on carbon (0.20 g). Thereafter, reaction mixture was stirred under hydrogen atmosphere at room temperature for 24h. After completion (monitored by LCMS), catalyst was removed by filtration on celite bed and rinsed with methanol, solvents were evaporated under reduced pressure and directly purified by prep HPLC (25-35% acetonitrile in water with 0.1% TFA) Fractions containing desired product were combined and lyophilized to dryness to afford (S)-3-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)-5-(methoxymethyl)midazolidi-2-one (**XC10**) as an off white solid. Yield: 0.070 g, 73.08 %; LCMS m/z 278.10 $[M+1]^+$. ^{1}H NMR (400 MHz, DMSO-d_6 with D_2O exchange) δ 4.65-4.59 (m,1H), 3.80-3.73 (m, 2H), 3.62-3.56 (m, 4H), 3.48-3.40 (m, 4H), 3.27 (s, 3H), 3.26-3.20 (m, 2H).

Synthesis of Compound **XC25**:





8β XC25

To a stirred solution of (2R,3R,4R)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran (**3,** 1.0 eq., 4.5 g, 9.75 mmol) and dimethyl (S)-2-hydroxysuccinate (2.0 eq., 3.16 g, 19.5 mmol) in anhydrous toluene (30 mL) under Ar, was added activated molecular sieves (3 Å, 1.50 g) and stirred for 1h at room temperature. Then, potassium *tert*-butoxide (1 M solution in THF, 4.88 mmol, 4.88 mL, 0.5 eq.,) was added at 0 °C and stirred for 4h at room temperature. Thereafter, acetic acid (0.5 mL) was added to quench the reaction, and molecular sieves were filtered off. The filtrate was concentrated under reduced pressure to get residue which was purified by silica gel flash column chromatography using 10% ethyl acetate in hexane to furnish dimethyl (2S)-2-(((3R,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-3-nitrotetrahydro-2H-pyran-2-yl)oxy)succinate (**4**) as colorless oil. Yield 3.1 g, 50.98%. LCMS m/z 641.32 $[M+18]^+$.

To a stirred solution of dimethyl (2S)-2-(((3R,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-3-nitrotetrahydro-2H-pyran-2-yl)oxy)succinate (**4,** 1.0 eq., 3.1 g, 3.48 mmol) in glacial acetic acid (15 mL), zinc (12.0 eq., 2.73g, 41.8 mmol) was added at room temperature and then refluxed for 6h. After completion (monitored by TLC), the reaction mixture was diluted with methanol and filtered over *celite* pad. The filtrate was evaporated to get crude dimethyl (2S)-2-(((3R,4R,5R,6R)-3-amino-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)succinate (**5**) as brown syrup. Yield: 2.0 g, crude. LCMS m/z 594.12 $[M+H]^+$.

The solution of dimethyl (2S)-2-(((4R,5R,6R)-3-amino-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-2-yl)oxy)succinate (**5,** 1.0 eq. 2.0 g, 3.37 mmol) in methanol (20 mL) was refluxed for 6h. The volatile was evaporated to get crude which was purified by silica gel flash column chromatography using 60-70% ethyl acetate in hexane to furnish methyl 2-((3S,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetate (**6**) as yellow sticky solid. Yield: 0.80 g, 42.28%; LCMS m/z 560.12 $[M-H]^-$.

To a stirred solution of methyl 2-((3S,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetate (**6,** 1.0 eq., 0.78 g, 1.39 mmol) in mixture

of tetrahydrofuran:methanol:water (7 mL, 4:2:1,v/v/v), lithium hydroxide monohydrate (1.1 eq., 0.064 g, 1.53 mmol) was added at 0 °C and stirred at room temperature for 3h. After completion, the reaction mixture was acidified with 1N HCl and extracted by dichloromethane (3x100 mL). Then organic part was washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated to give crude 2-((3S,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetic acid (**7**) which was used for next step without further purification. Yield: 0.530 g, crude; LCMS m/z 546.12 $[M-H]^+$.

To a stirred solution 2-((3S,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetic acid (**7,** 1.0 eq. 0.530 g, 0.968 mmol) in dry N,N-dimethylformamide (9 mL), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) (1.2 eq., 0.442 g, 1.16 mmol), N,N-diisopropylethylamine, (3.0 eq., 0.521 mL, 2.9 mmol) were added at 0 °C. Then, tert-butyl (4-aminobutyl)carbamate (1.1 eq. 0.20 g, 1.16 mmol) was added, and stirred the reaction mixture at room temperature for 12 h. After completion (monitored by TLC), water was added, and extracted with dichloromethane. The organic part was washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated to give crude which was purified by pre-HPLC (60% acetonitrile in water with 0.1% acetic acid) to give tert-butyl (4-(2-((3S,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetamido)butyl)carbamate (mixture of both isomer) which was further passed through Chiral SFC to give tert-butyl (4-(2-((3S,4aR,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetamido)butyl)carbamate (8α, 0.120 g, 18%) and tert-butyl (4-(2-((3S,4aS,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetamido)butyl)carbamate (**8β**, 0.015 g. 3%).

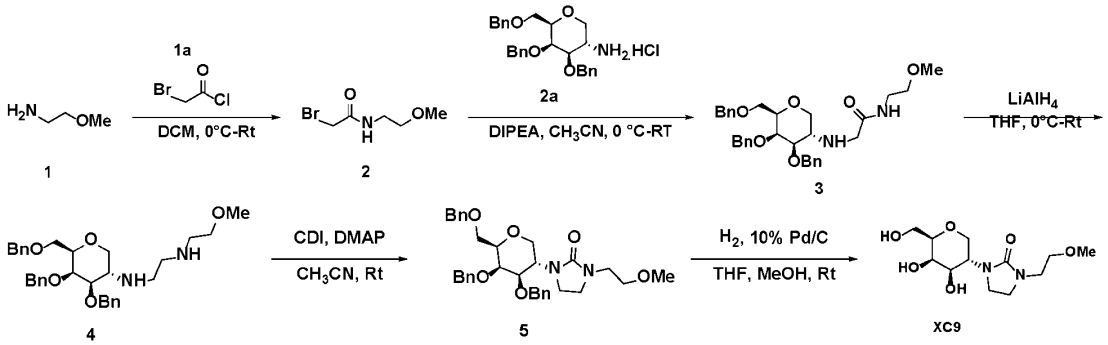
(8α) ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44 (d, J = 4.8 Hz, 1H), 7.88 (t, J = 4.8 Hz, 1H), 7.44 (d, J = 7.2 Hz, 2H), 7.36-7.22 (m, 13H), 6.75 (t, J = 5.6 Hz, 1H), 5.29 (d, J = 2.4 Hz, 1H), 4.79-4.70 (m, 2H), 4.65-4.62 (m, 1H), 4.60-4.57 (m, 1H), 4.51-4.41 (m, 3H), 4.01 (s, 2H), 3.81 (d, J = 10 Hz, 1H), 3.56-3.48 (m, 3H), 3.05-2.99 (m, 2H), 2.88-2.87 (m, 2H), 2.68-2.62 (m, 1H), 2.43-2.32 (m, 1H), 1.36 (s, 13H).

(8β) ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.31 (d, J = 4.0 Hz, 1H), 7.88 (t, J = 6.0 Hz, 1H), 7.53 (d, J = *9.2* Hz, 2H), 7.49-7.20 (m, 13H), 6.77 (s, 1H), 5.25 (brs, 1H), 4.81-4.78 (m, 1H), 4.73-4.65 (m, 2H), 4.54-4.40 (m, 4H), 4.03-3.97 (m, 3H), 3.57-3.44 (m,, 3H), 3.16-3.02 (m, 2H), 2.90 (s, 2H), 2.66-2.51 (m, 2H), 1.36 (s, 14H), 1.21 (s, 1H).

Method for Chiral Separation: Column Name : Chiralpak IB-N5 (250*4.6)mm, 5um; Mobile Phase : CO2:0.1%Ipamine in IPA 60:40; Flow Rate : 3.0 mL/min Column; Temperature : 40°C.

To a solution of tert-butyl (4-(2-((3S,4aS,6R,7R,8R,8aR)-7,8-bis(benzyloxy)-6-((benzyloxy)methyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetamido)butyl)carbamate (**8β**, 0.500 g, 0.697 mmol, 1.0 cq.) in methanol (10 mL), 10% Pd/C (0.900 g) was added and stirred at room temperature under H_2 gas (balloon pressure) for 48h. After completion (monitor by LCMS), the reaction mixture was filtered on celite pad and washed with methanol. The filtrate was evaporated under reduced pressure to give crude which was again treated with a mixture trifluoroacetic acid and dichloromethane (10 ml, 1:1, v/v) and stirred for 1h at room temperature. Thereafter, the reaction mixture was purified by prep-HPLC (20-30% acetonitrile in water with 0.1% TFA) to give N-(4-aminobutyl)-2-((3S,4aS,6R,7R,8R,8aR)-7,8-dihydroxy-6-(hydroxymethyl)-2-oxohexahydro-1H,6H-pyrano[2,3-b][1,4]oxazin-3-yl)acetamide (**XC25**) as colorless semi solid. Yield: 0.060 g, 35%; LCMS 348.30 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6 with D_2O exchange) δ 5.17 (d, J = 2.8 Hz, 1H), 4.48-4.46 (m, 1H), 3.76 (t, J = 6.4 Hz, 1H), 3.72-3.67 (m, 2H), 3.52-3.43 (m, 2H), 3.24-3.09 (m, 2H), 3.27 (dd, J = 9.6, 2.8 Hz, 1H), 3.05 (t, J = 6.8 Hz, 1H), 2.75 (t, J = 7.2 Hz, 1H), 2.61(dd, J = 14.8, 3.2 Hz, 1H), 1.52-1.46 (m, 2H), 1.44-1.39 (m, 2H).

Synthesis of **XC9**:



To a stirred solution of 2-methoxyethan-1-amine (1.0 eq., 1.16 mL, 13.3 mmol) in dichloromethane (50 mL), 2-bromoacetyl chloride (0.7 eq,. 0.772 mL, 9.32 mmol) was added dropwise at 0°C under N_2 and then allowed to stir at room temperature for 12h. After completion (monitored by TLC), reaction mixture was quenched by addition of ice cold water followed by extracted with dichloromethane. Organic layer was then washed with brine, dried over anhydrous sodium sulfate and concentrated under vacuum to get 2-bromo-N-(2-methoxyethyl)acetamide (1.0 g, 5.1 mmol) as colorless liquid. Yield: 1.0 g (crude), 38.31 %; ^1H NMR (400 MHz, DMSO-d_6): δ 8.30 (bs, 1H), 4.05 (d, J = 7.20 Hz, 1H), 3.85 (s, 1H), 3.36-3.52 (m, 2H), 3.21-3.35 (m, 5H).

A solution of 2-bromo-N-(2-methoxyethyl)acetamide (2.0 eq., 0.181 g 0.923 mmol) and (3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-amine hydrochloride (1.0 eq., 0.2 g 0.461 mmol) in acetonitrile (5.0 mL) was cooled at 0 °C, *N,N*-diisopropylethylamine (0.403 mL, 5 0 eq., 1.02 mmol) was added and the reaction mixture was stirred at room temperature for 16h. After completion (monitored by LCMS), reaction mixture was concentrated under vacuum to get crude product which was re-dissolved in ethyl acetate, washed with ice cold water. The organic layer was dried over anhydrous sodium sulfate, concentrated to get crude which was purified by silica gel flash column chromatography using 2-5% methanol in dichloromethane to afford 2-(((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)amino)-N-(2-methoxyethyl)acetamide **(3)** as a green oily liquid. Yield: 0.120 g, 47.41 %; LCMS m/z 549.10 $[M+1]^+$.

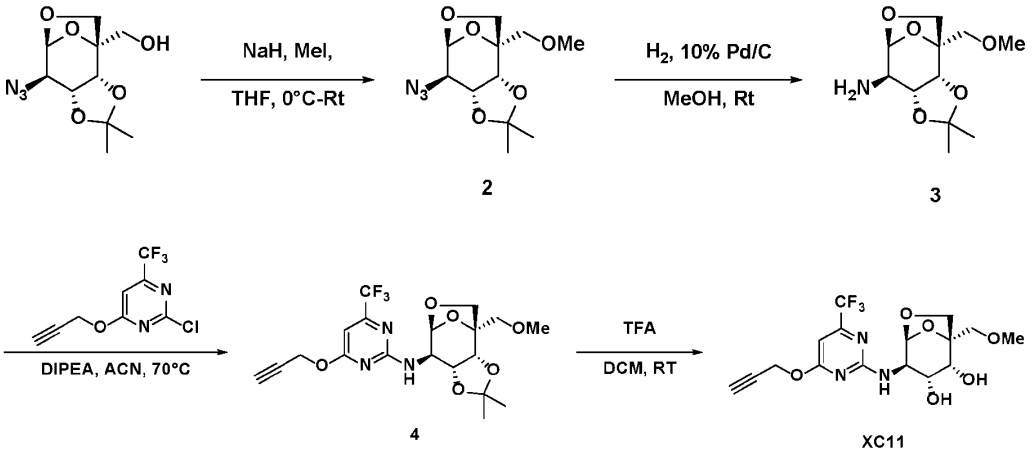
A solution of 2-(((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)amino)-N-(2-methoxyethyl)acetamide **(3,** 1.0 eq, 0.220 g, 0.401mmol) in tetrahydrofuran (4.0 mL) was cooled at 0 °C, lithium aluminum hydride (4 0 eq., 0.656 mL, 1.31 mmol, 1M in THF) was added and reaction mixture was stirred at room temperature for 12 h. After completion (monitored by LCMS), reaction mixture was quenched by 15 % sodium hydroxide solution, brine solution was added, then extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated to give Crude N1-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)-N2-(2-methoxyethyl)ethane-1,2-diamine **(4)** as brown syrup which was used for next reaction without further purification. Yield: 0.170 g (Crude); LCMS m/z 535.10 $[M+1]^+$.

To a solution of N1-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)-N2-(2-methoxyethyl)ethane-1,2-diamine **(4,** 1.0 eq., 0.11 g, 0.206 mmol) in acetonitrile (2.75 mL), 1-(1H-imidazole-1-carbonyl)-1H-imidazole (0.05 g, 1.5 eq., 0.309 mmol) and *N,N*-dimethylpyridin-4-amine (0.1 eq., 0.0025 g 0.0020 mmol) were added and reaction mixture was stirred at room temperature for 16h. After completion (monitored by LCMS), reaction mixture was concentrated to give crude residue which was purified by flash column chromatography on silica gel using 50% ethyl acetate in hexane to afford a 1-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)-3-(2-methoxyethyl)midazolidine-2-one **(5)** as a colorless liquid. Yield: 0.080 g, 69.36 %; LCMS m/z 561.15 $[M+1]^+$.

To a stirred solution of 1-((3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)tetrahydro-2H-pyran-3-yl)-3-(2-methoxyethyl)midazolidine-2-one **(5,** 1 eq, 0.047 g 0.0838 mmol) in methanol (3 mL) were added 20% Palladium hydroxide on carbon (0.05 g) and 10% palladium on carbon (0.5 g). Then reaction mixture was purged with hydrogen gas and stirred under hydrogen atmosphere. After completion, the reaction mixture was filtered through celite bed and rinsed with methanol The filtrate was

concentrated under reduce pressure to afford a crude which was purified by prep HPLC (20-30% acetonitrile in water with 0.1% TFA) to afford 1-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)-3-(2-methoxyethyl)midazolidine-2-one (Cpd. No. **XC9**) as colorless semi solid. Yield: 0.003 g, 12.5 %; LCMS m/z 291.15 $[M+1]^+$; ^{1}H NMR (400 MHz, DMSO-d_6 with D_2O exchange): δ 3.85-3.75 (m, 1H), 3.71 (bs, 1H), 3.61-3.53 (m, 1H), 3.45 (d, J = 6.0 Hz, 1H), 3.38 (t, J = 5.6 Hz, 1H), 3.30-3.16 (m, 7H).

Synthesis of **XC11**:



To a stirred solution of ((3aR,4S,7S,8R,8aR)-8-azido-2,2-dimethyltetrahydro-4,7-epoxy[1,3]dioxolo[4,5-d]oxepin-4(5H)-yl)methanol (1.0 eq., 0.430 g., 1.67 mmol.) in tetrahydrofuran (5 mL) was add sodium hydride (1.5 eq., 0.1 g, 2.51 mmol.) at 0° C and stirred for 30 minutes, then methyl iodide (5.0 eq., 0.520 ml.,8.36 mmol) was added and the mixture was stirred at room temperature for 2 h. After completion (monitored by TLC), reaction mixture was quenched with cold water and extracted with dichloromethane (2-3 times). The organic layer was dried over anhydrous sodium sulphate, filtered, and concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-60 % ethyl acetate in hexane to afford (3aR,4S,7S,8R,8aR)-8-azido-4-(methoxymethyl)-2,2-dimethylhexahydro-4,7-epoxy[1,3]dioxolo[4,5-d]oxepine (**2**) as colorless liquid. Yield: 0.30 g, 66.16%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.43 (d, J = 2.0 Hz, 1H), 4.29 (d, J = 4.8 Hz, 2H), 3.91 (d, J = 7.6 Hz, 1H), 3.77 (d, J = 10.0 Hz, 1H), 3.66 (d, J = 7.6 Hz, 1H), 3.58 (d, J = 10.0 Hz, 1H), 3.39 (t, J = 4.8 Hz, 1H), 3.32 (d, J = 6.0 Hz, 3H), 1.45 (s, 3H), 1.31 (s, 3H).

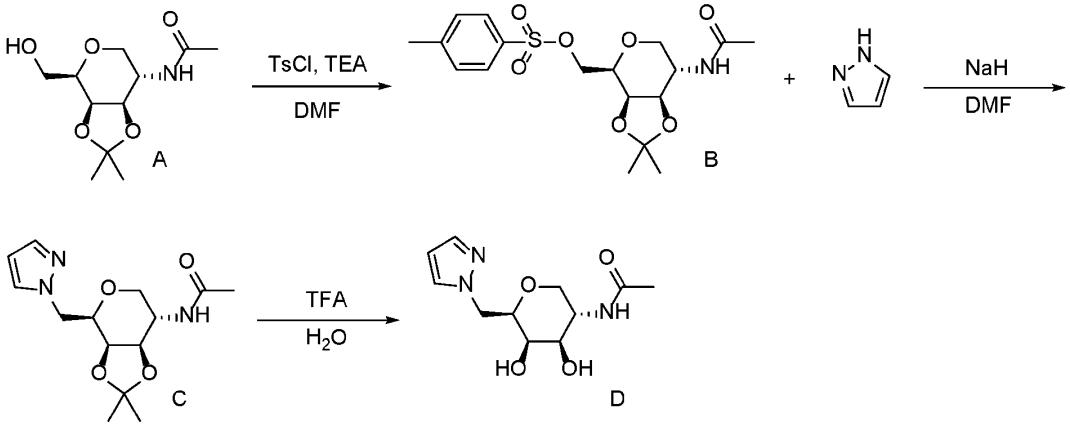
To a stirred solution of (3aR,4S,7S,8R,8aR)-8-azido-4-(methoxymethyl)-2,2-dimethylhexahydro-4,7-epoxy[1,3]dioxolo[4,5-d]oxepine (**2,** 1.0 eq., 0.3 g., 1.11 mmol) in methanol (5.0 mL) was added 10% palladium on carbon (0.12 g) and stirred under hydrogen atmosphere. After completion (monitored by

TLC and LCMS), the reaction mixture was filtered through celite bed and rinsed with methanol. The filtrate was concentrated under vacuum to afford (3aR,4S,7S,8R,8aR)-4-(methoxymethyl)-2,2-dimethylhexahydro-4,7-epoxy[1,3]dioxolo[4,5-d]oxepin-8-amine (**3**) as a light yellow viscous liquid. Yield: 0.070 g, 25.81 %; LCMS m/z 246.10 $[M+1]^+$.

To a stirred solution of (3aR,4S,7S,8R,8aR)-4-(methoxymethyl)-2,2-dimethylhexahydro-4,7-epoxy[1,3]dioxolo[4,5-d]oxepin-8-amine (**3**, 1.0 eq., 0.070 g.,0.285 mmol) and 2-chloro-4-(prop-2-yn-1-yloxy)-6-(trifluoromethyl)pyrimidine (1.0 eq., 0.0675 g 0.285 mmol) in acetonitrile (1.0 mL) was added *N,N*-diisopropylethylamine (5.0 eq., 0.249 mL 1.43 mmol) at room temperature, and then heated at 70°C for 16h. After completion (monitored by LCMS), reaction mixture was concentrated to get crude. Which was purified by column chromatography using silica gel (100-200 mesh) and 0-10 % methanol in DCM to afford N-((3aR,4S,7S,8R,8aR)-4-(methoxymethyl)-2,2-dimethylhexahydro-4,7-epoxy[1,3]dioxolo[4,5-d]oxepin-8-yl)-4-(prop-2-yn-1-yloxy)-6-(trifluoromethyl)pyrimidin-2-amine (**4**)as a white solid. Yield: 0.070 g, 55.07%; LCMS m/z 446.05 $[M+1]^+$.

A solution of N-((3aR,4S,7S,8R,8aR)-4-(methoxymethyl)-2,2-dimethylhexahydro-4,7-epoxy[1,3]dioxolo[4,5-d]oxepin-8-yl)-4-(prop-2-yn-1-yloxy)-6-(trifluoromethyl)pyrimidin-2-amine (1.0 eq., 0.080 g, 0.180 mmol) in dichloromethane (1.0 mL) was cooled at 0 °C, trifluoroacetic acid (1.0 mL) was added and reaction mixture was stirred at room temperature for 1 h. After completion, reaction mixture was purified by prep HPLC (45-55% acetonitrile in H_2O with 0.1% TFA). Fractions containing desired compound were collected and lyophilized to afford (1S,2R,3R,4R,5S)-1-(methoxymethyl)-4-((4-(prop-2-yn-1-yloxy)-6-(trifluoromethyl)pyrimidin-2-yl)amino)-6,8-dioxabicyclo[3.2.1]octane-2,3-diol (Cpd. No. **XC11**) as an off white solid. Yield: 0.0013 g, 1.37 %; m/z 406.0 $[M+1]^+$.

Synthesis of **XC17**:

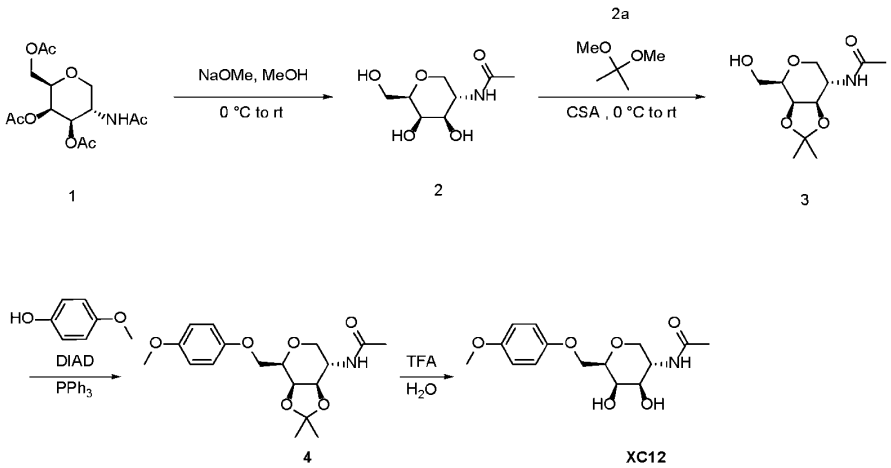


A solution of **A** (1.0 eq, 80.0 mg, 0.326 mmol) in THF (0.815 mL) was cooled in an ice bath then triethylamine (2.2 eq, 100 mL, 0.718 mmol) was added followed by *p*-toluenesulfonyl chloride (2.0 eq, 124 mg, 0.652 mmol). The reaction was stirred at room temperature for 1 hour then 50 °C for 18 h. The crude reaction was adsorbed to celite then purified by silica gel chromatography (0-20% MeOH in DCM) to give ((3aR,4R,7S,7aR)-7-acetamido-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-4-yl)methyl 4-methylbenzenesulfonate. Yield: 90 mg, 69%. LCMS *m/z* 400.1 $[M+H]^+$.

A mixture of sodium hydride (3.0 eq, 4.5 mg, 0.113 mmol) and 1H-pyrazole (3.2 eq, 8.2 mg, 0.120 mmol) under nitrogen was dissolved in DMF (0.188 mL) then ((3aR,4R,7S,7aR)-7-acetamido-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-4-yl)methyl 4-methylbenzenesulfonate (1.00 eq, 15.0 mg, 0.0376 mmol) was added and the reaction was stirred at room temperature for 3 h then 19 h at 80 °C. The reaction was diluted with DCM (2 mL) and water (3 mL). The organic layer was washed with water (5 mL) then dried over Na_2SO_4, filtered, and concentrated to give N-((3aS,4R,7S,7aR)-4-((1H-pyrazol-1-yl)methyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide as a solid. The residue was used in the next step without further purification. LCMS *m/z* 296.2 $[M+H]^+$.

N-((3aS,4R,7S,7aR)-4-((1H-pyrazol-1-yl)methyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide (1.0 eq, 7.0 mg, 0.0237 mmol) was dissolved in water (150 mL) and trifluoroacetic acid (88.8 eq, 150 mL, 2.10 mmol). After 90 minutes, the reaction was concentrated under vacuum then diluted further with water and MeOH before being purified by reversed-phase HPLC (5-30% acetonitrile in water w/0.1% FA) to give N-((3S,4R,5R,6R)-6-((1H-pyrazol-1-yl)methyl)-4,5-dihydroxytetrahydro-2H-pyran-3-yl)acetamide (XC17). Yield: 1.0 mg, 16.5%. LCMS *m/z* 312.1 $[M+H]^+$.

Synthesis of **XC12**:



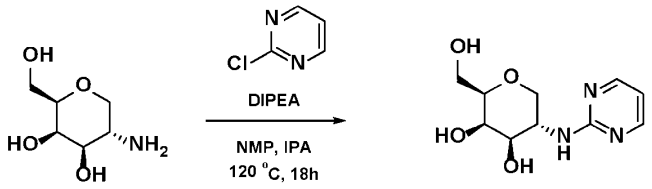
To a stirred solution of (2R,3R,4R,5S)-5-acetamido-2-(acetoxymethyl)tetrahydro-2H-pyran-3,4-diyl diacetate (**example 48 48-3**, 1.0 eq, 2.0 g, 6.04 mmol) in methanol (20 mL), sodium methoxide 25% w/v in methanol (0.2 eq, 10.3 mL, 179 mmol) was added drop-wise at 0 °C. The reaction mixture was allowed to stir at room temperature for 2 h. After completion (monitored by ELSD), the reaction mixture was neutralized using Dowex-hydrogen form (200-400 mesh) (up to pH-7). The reaction mixture was filtered, organic was concentrated under reduced pressure to get crude. The crude was used for the next step. Yield: 1.1 g (crude).

To a solution of N-((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (1.1 g, 1.0 eq, 5.36 mmol) in 2,2-dimethoxypropane (20 mL) was added camphor sulphonic acid (0.125 g, 0.1 eq, 0.536 mmol) and the reaction mixture was stirred at room temperature for 3 h. After completion, the reaction mixture was quenched with triethylamine (2.26 mL, 3.0 eq, 16.1 mmol) and concentrated under reduced pressure to obtain crude. The crude was purified by prep HPLC (10-25% acetonitrile in water with 0.05% ammonium acetate). Fractions containing the desired product were combined and lyophilized to dryness to afford N-((3aR,4R,7S,7aR)-4-(hydroxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide (**3**) as a white solid. Yield: 0.582 g, 44.27%; LCMS m/z 246.10 [M+1]+; 1H NMR (400 MHz, MeOD) δ 4.20 (d, *J*=0.8Hz, 1H), 4.00-3.97 (m, 2H), 3.85-3.81 (m, 1H), 3.73-3.69 (m, 3H), 3.04 (t, *J*=10.8 Hz, 1H), 1.95 (s, 3H), 1.48 (s, 3H), 1.32 (s, 3H).

A solution of triphenylphosphine (1.5 eq, 27.3 mg, 0.104 mmol) in THF (0.693 mL) was cooled in an ice bath before adding diisopropyl azodicarboxylate (1.2 eq, 16 uL, 0.0832 mmol). After 5 minutes, N-((3aR,4R,7S,7aR)-4-(hydroxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide (1.00 eq, 17.0 mg, 0.0693 mmol) was added and after another 10 minutes, 4-methoxyphenol (1.5 eq, 12.9 mg, 0.104 mmol) was added and the reaction was stirred at room temperature for 1 hour then at 50 °C for 18 h. The reaction was purified directly by reversed-phase HPLC (10-50% acetonitrile in water w/0.1% FA) to give N-((3aR,4R,7S,7aR)-4-((4-methoxyphenoxy)methyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide. LCMS m/z 351.5 $[M+H]^+$.

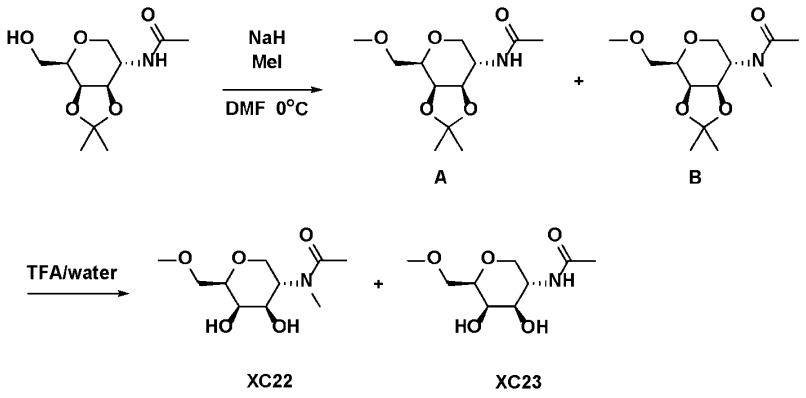
A solution of N-((3aR,4R,7S,7aR)-4-((4-methoxyphenoxy)methyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide in a solution of TFA/water (1:1) was stirred at room temperature for 1 hour. The reaction was purified by reversed-phase HPLC (5-50% acetonitrile in water with 0.1% FA) to give N-((3S,4R,5R,6R)-4,5-dihydroxy-6-((4-methoxyphenoxy)methyl)tetrahydro-2H-pyran-3-yl)acetamide, **XC12**, as a white solid. Yield: 1.1 mg, 5%. LCMS m/z 312.1 $[M+H]^+$.

Synthesis of **XC15**:



A solution of (2R,3R,4R,5S)-5-amino-2-(hydroxymethyl)tetrahydro-2H-pyran-3,4-diol;hydrochloride (1.00 eq, 10.0 mg, 0.0501 mmol), 2-chloropyrimidine (3.00 eq, 17.2 mg, 0.150 mmol) and DIPEA (4.00 eq, 35 mL, 0.200 mmol) in IPA (0.251 mL) and NMP (0.251 mL) was heated to 120 °C for 18 h. The reaction was diluted with water then purified by reversed-phase HPLC (4-100% acetonitrile in water w 200 mM NH4OH) to give (2R,3R,4R,5S)-2-(hydroxymethyl)-5-(pyrimidin-2-ylamino)tetrahydro-2H-pyran-3,4-diol (**XC15**). Yield: 1.2 mg, 10%. LCMS *m/z* 242.1 $[M+H]^+$.

Synthesis of **XC22** and **XC23**:



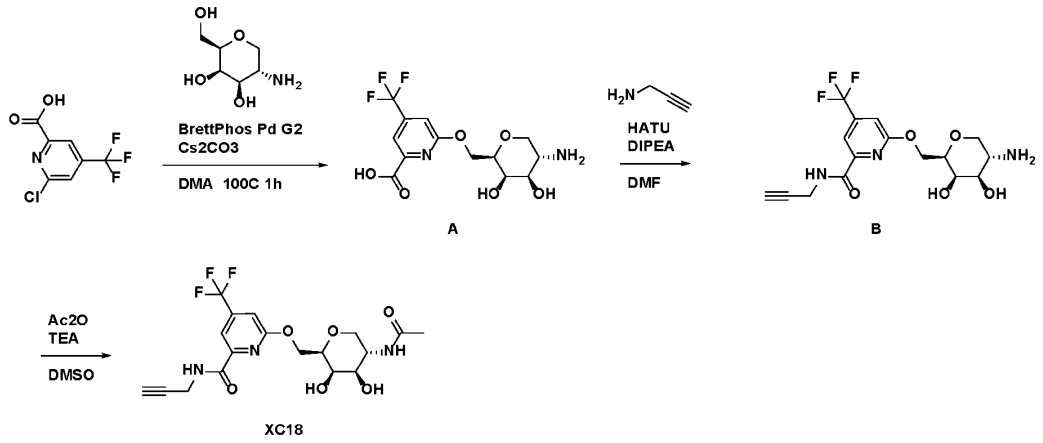
A solution of N-((3aR,4R,7S,7aR)-4-(hydroxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide (1.0 eq, 30.0 mg, 0.122 mmol) and sodium hydride (2.0 eq, 9.8 mg, 0.245 mmol) in DMF (0.612 mL) was cooled in an ice bath before adding iodomethane (1.5 eq, 11 mL, 0.183 mmol) then removing the ice bath. After 1 hour, the reaction was filtered then purified by RPHPLC (10-50% acetonitrile in water) to give N-((3aR,4R,7S,7aR)-4-(methoxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide (22 mg, 0.0848 mmol, 69 % yield) and N-((3aR,4R,7S,7aR)-4-(methoxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)-N-methylacetamide. Yield: 7.0 mg, 21% and 22 mg, 69% respectively. LCMS m/z 274.0 $[M+H]^+$.

A solution of N-((3aR,4R,7S,7aR)-4-(methoxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)-N-methylacetamide (1.0 eq, 7.0 mg, 0.0256 mmol) in water (300 mL) was

treated with trifluoroacetic acid (10.0 eq, 0.018 mL, 0.256 mmol). The reaction was filtered then purified by reversed-phase HPLC (5-50% acetonitrile in water) to give N-((3S,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)tetrahydro-2H-pyran-3-yl)-N-methylacetamide (**XC22**). Yield: 3.4 mg, 57 %. LCMS m/z 234.0 $[M+H]^+$.

A solution of N-((3aR,4R,7S,7aR)-4-(methoxymethyl)-2,2-dimethyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)acetamide (1.0 eq, 22.0 mg, 0.0848 mmol) in water (300 mL) was treated with formic acid (16.0 eq, 51 mL, 1.36 mmol) and after 1h, some starting material was remaining so trifluoroacetic acid (8.4 eq, 51 mL, 0.716 mmol) was added. The reaction was filtered then purified by reversed-phase HPLC (5-50% acetonitrile in water) to give N-((3S,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (**XC23**). Yield: 6.6 mg, 35%. LCMS m/z 234.0 $[M+H]^+$.

Synthesis of **XC18**:

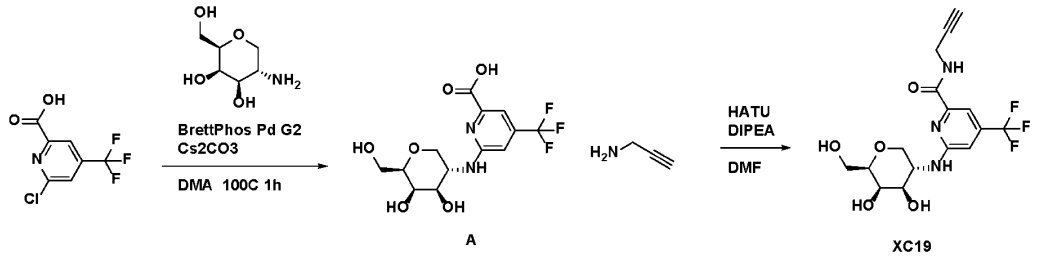


A vial containing (2R,3R,4R,5S)-5-amino-2-(hydroxymethyl)tetrahydropyran-3,4-diol;hydrochloride (1.5 eq, 53.1 mg, 0.266 mmol), 6-chloro-4-(trifluoromethyl)picolinic acid (1.0 eq, 40.0 mg, 0.177 mmol) and BrettPhos Pd G3 (0.2 eq, 32.2 mg, 0.0355 mmol) was placed under nitrogen in a dry box then anhydrous DMA (1.773 mL) was added followed by an anhydrous solution of sodium tert-butoxide solution (2M in THF, 4.0 eq, 355 mL, 0.709 mmol) and the reaction was sealed then heated to 100 °C for 2h. The reaction was diluted with water and DMSO then purified by reversed-phase HPLC (3-70% acetonitrile in water w/0.1% TFA) to give 6-[[(3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]amino]-4-(trifluoromethyl)pyridine-2-carboxylic acid as a mixture of regio-isomers. Yield: 15 mg, 24%. LCMS *m/z* 353.1 $[M+H]^+$.

A solution of the mixture from the synthesis of A (15 mg), propargylamine (3.00 eq, 5.5 mL, 0.0852 mmol), and DIPEA (1.0 eq, 2.9 mg, 0.0284 mmol) in DMSO (0.600 mL) was treated with HATU (1.2 eq, 13.0 mg, 0.0341 mmol) at room temperature. After 2 h, the solution was purified by reversed-phase HPLC (10-50% acetonitrile in water w/0.1% TFA) to give 6-(((2R,3R,4R,5S)-5-amino-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methoxy)-N-(prop-2-yn-1-yl)-4-(trifluoromethyl)picolinamide. Yield: 3.3 mg, 30%. LCMS *m/z* 390.0 $[M+H]^-$.

A solution of 6-[[(2R,3R,4R,5S)-5-amino-3,4-dihydroxy-tetrahydropyran-2-yl]methoxy]-N-prop-2-ynyl-4-(trifluoromethyl)pyridine-2-carboxamide (1.0 eq, 3.3 mg, 0.00848 mmol) in DMSO (0.500 mL) was treated with triethylamine (3.0 eq, 3.5 mL, 0.0254 mmol) followed by acetic anhydride (1.0 eq, 0.80 mL, 0.00848 mmol) and the reaction was stirred at room temperature. After 2 h, the reaction was purified by reversed-phase HPLC (10-50% acetonitrile in water w/0.1% TFA) to give 6-[[(2R,3R,4R,5S)-5-acetamido-3,4-dihydroxy-tetrahydropyran-2-yl]methoxy]-N-prop-2-ynyl-4-(trifluoromethyl)pyridine-2-carboxamide (**XC18**). Yield: 1.1 mg, 31%. LCMS *m/z* 432.2 $[M+H]^+$.

Synthesis of **XC19**:

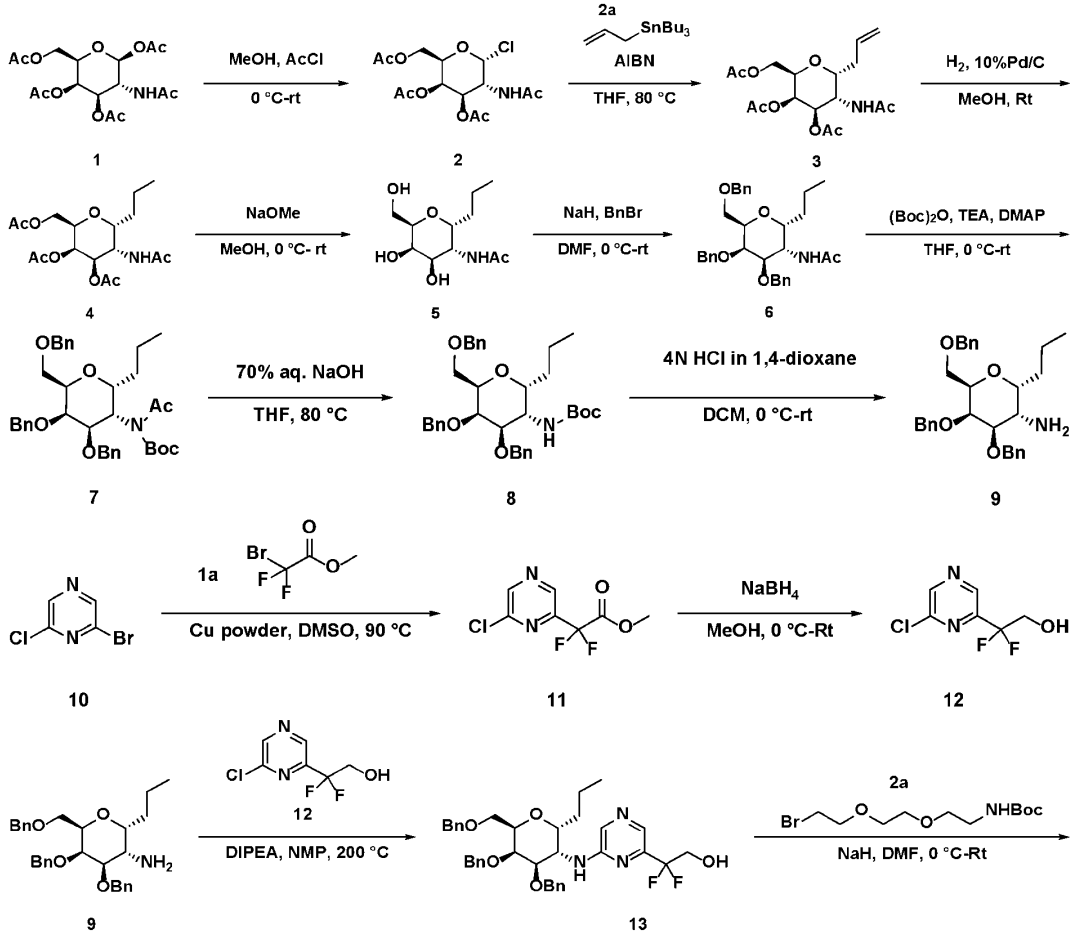


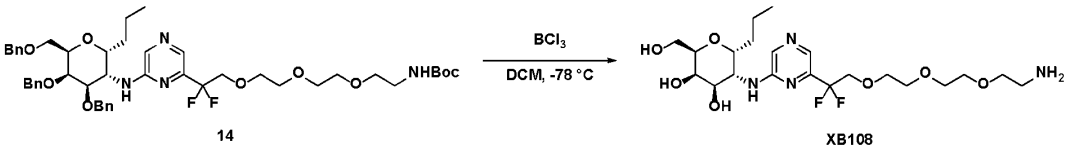
A vial containing (2R,3R,4R,5S)-5-amino-2-(hydroxymethyl)tetrahydropyran-3,4-diol;hydrochloride (example **48-4**, 1.5 eq, 53.1 mg, 0.266 mmol), 6-chloro-4-(trifluoromethyl)picolinic acid (1.0 eq, 40.0 mg, 0.177 mmol) and BrettPhos Pd G3 (0.2 eq, 32.2 mg, 0.0355 mmol) was placed under nitrogen in a dry box then anhydrous DMA (1.773 mL) was added followed by an anhydrous solution of sodium tert-butoxide (2M in THF, 4.0 eq, 355 mL, 0.709 mmol) and the reaction was sealed then heated to 100 °C for 2h. The reaction was diluted with water and DMSO then purified by reversed-phase HPLC (3-70% acetonitrile in water w/0.1% TFA) to give 6-[[(3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]amino]-4-(trifluoromethyl)pyridine-2-carboxylic acid as a mixture of regio-isomers. Yield: 15 mg, 24%. LCMS *m/z* 353.1 $[M+H]^+$.

A solution of propargylamine (3.00 eq, 5.5 mL, 0.0852 mmol), 6-[[(3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]amino]-4-(trifluoromethyl)pyridine-2-carboxylic acid (1.0 eq, 10.0 mg, 0.0284 mmol) and DIPEA (1.0 eq, 2.9 mg, 0.0284 mmol) in DMSO (0.600 mL) was

treated with HATU (1.2 eq, 13.0 mg, 0.0341 mmol) at room temperature. After 2 h, the solution was purified by reversed-phase HPLC (10-50% acetonitrile in water w/0.1% TFA) to give 6-[[(3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]amino]-N-prop-2-ynyl-4-(trifluoromethyl)pyridine-2-carboxamide (**XC19**). Yield: 1.1 mg, 10%. LCMS *m/z* 390.0 $[M+H]^+$.

Synthesis of (2R,3R,4R,5R,6R)-5-((6-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-1,1-difluoroethyl)pyrazin-2-yl)amino)-2-(hydroxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol (XB108):





14 XB108

Synthesis of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-chlorotetrahydro-2H-pyran-3,4-diyl diacetate (**2**)

A solution of (2S,3R,4R,5R,6R)-3-acetamido-6-(acetoxymethyl)tetrahydro-2H-pyran-2,4,5-triyl triacetate (**1**, 40 g, 1.0 eq., 103 mmol) in acetyl chloride (120 mL) was cooled to 0 °C. To this, dry methanol (10 mL) was added slowly, and the resultant reaction mixture was stirred at room temperature for 12h. After completion, the reaction mixture was concentrated under reduced pressure, and recrystallized with ethyl acetate and ether (9:1) to afford (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-chlorotetrahydro-2H-pyran-3,4-diyl diacetate (**2**) as a white solid. Yield: 32.0 g, 85.0%; LCMS: m/z 366.1 $[M+H]^+$

Synthesis of (2R,3R,4R,5S,6R)-5-acetamido-2-(acetoxymethyl)-6-allyltetrahydro-2H-pyran-3,4-diyl diacetate (**3**)

To a solution of (2R,3R,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-chlorotetrahydro-2H-pyran-3,4-diyl diacetate (**2**, 17.0 g, 46.5 mmol) and allyltributylstannane (100.8 mL, 7.0 eq., 326.0 mmol) in tetrahydrofuran (150 mL) was added azobisisobutyronitrile (2.29 g, 0.3 eq., 13.9 mmol). The resultant reaction mixture was purged with N_2 gas, and reflux at 80° C for 8h. After completion, tetrahydrofuran was removed under reduced pressure and the residue was partitioned between acetonitrile (120 mL) and pentane (200 mL). The acetonitrile layer was extracted with additional pentane (3x100 mL) to remove organotin compounds, and then concentrated to obtained crude which was purified by silica gel flash chromatography using 50-60% ethyl acetate in heptane as an eluent to afford (2R,3R,4R,5S,6R)-5-acetamido-2-(acetoxymethyl)-6-allyltetrahydro-2H-pyran-3,4-diyl diacetate (**3**) as off-white semi solid. Yield: 10.2 g, 59.0%; LCMS: m/z 372.4 $[M+H]^+$

Synthesis of (2R,3R,4R,5S,6R)-5-acetamido-2-(acetoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diyl diacetate (**4**)

To a stirred solution of (2R,3R,4R,5S,6R)-5-acetamido-2-(acetoxymethyl)-6-allyltetrahydro-2H-pyran-3,4-diyl diacetate (**3**, 8.4 g, 22.6 mmol) in methanol (80 mL) was added 10% palladium on carbon (5.0 g) and the resultant reaction mixture was stirred under hydrogen gas atmosphere (balloon pressure) at room temperature for 1h. After completion, the reaction mixture was filtered through celite, and the filtrate was concentrated under reduced pressure to afford (2R,3R,4R,5S,6R)-5-acetamido-2-(acetoxymethyl)-6-

propyltetrahydro-2H-pyran-3,4-diyl diacetate (**4**) as colorless viscous solid. Yield: 8.20 g, 97.1%; LCMS m/z 374.5 $[M+H]^+$.

Synthesis of N-((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)acetamide (**5**)

A solution of (2R,3R,4R,5S,6R)-5-acetamido-2-(acetoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diyl diacetate (**4**, 8.4 g, 22.5 mmol) in methanol (80 mL) was cooled at 0 °C. To this, sodium methoxide solution (1.54 mL, 0.3 eq., 6.75 mmol) in methanol (25 wt.%) was added and the reaction mixture was stirred at room temperature for 2h. After completion, reaction mixture was neutralized with Dowex 50WX8 hydrogen form (200-400 mesh) and filtered through sintered funnel (without celite). The filtrate was concentrated, washed with diethyl ether and dried to afford N-((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)acetamide (**5**) as an colourless viscous liquid. Yield: 4.80 g, 86.28%; LCMS m/z 248.1 $[M+H]^+$

Synthesis of N-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)acetamide (**6**)

A solution of N-((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)acetamide (**5**, 4.8 g, 19.4 mmol) in N,N-dimethylformamide (20 mL) was cooled to 0 °C, and sodium hydride (1.4 g, 3 eq., 58.2 mmol) was added in portion keeping temperature constant for 30 min. To this, benzyl bromide (6.92 mL, 3 eq., 58.2 mmol) was added and the resultant reaction mixture was stirred at room temperature for 16h. After completion, ice cooled water was added to the reaction mixture and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate, filtered and concentrated to get crude. Crude was purified by flash chromatography (Silica Gel: 100-200 mesh, 5-20 % ethyl acetate in heptane) to afford N-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)acetamide (**6**) as white solid. Yield: 6.80 g, 67.6%; LCMS: m/z 518.7 $[M+H]^+$

Synthesis of tert-butyl acetyl((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**7**)

A solution of N-((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)acetamide (**6**, 6.80 g, 13.1 mmol) in tetrahydrofuran (70 mL) was cooled to 0 °C. To this, N,N-dimethylpyridin-4-amine (0.321 g, 0.2 eq., 2.63 mmol), triethylamine (5.54 mL, 3 eq., 39.4 mmol) and di-tert-butyl dicarbonate (60.4 mL, 20.0 eq., 263 mmol) was added and the resultant reaction mixture was stirred at room temperature for 16h. After completion, the mixture was concentrated under reduced pressure to give crude product which was purified by flash chromatography (Silica gel: 100-200 mesh,

and 0-15 % ethyl acetate in heptane) to afford tert-butyl acetyl((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**7**) as yellow coloured sticky liquid. Yield: 7.0 g, 86.2%; LCMS: m/z 618.5 $[M+H]^+$

Synthesis of tert-butyl ((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**8**)

To a solution of tert-butyl acetyl((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**7**, 7.0 g, 11.3 mmol) in tetrahydrofuran (30 mL) was added 70% aqueous solution of sodium hydroxide (28 mL) at room temperature and the reaction mixture was stirred at 60° C for 12h. After completion, reaction mixture was diluted with water and extracted with ethyl acetate to give crude product, which was purified by flash chromatography (Silica gel: 100-200 mesh, and 30-40% Ethyl acetate in heptane) to afford tert-butyl ((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**8**) as white solid. Yield: 5.2 g, 79.7%; LCMS: m/z 593.5 $[M+18]^+$

Synthesis of (2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-amine (**9**)

A solution of tert-butyl ((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**8**, 1.20 g, 2.02 mmol) in dichloromethane (10 mL) was cooled to 0 °C. To this, 4N hydrochloric acid in 1,4-dioxane (15 mL) was added, and the reaction mixture was stirred at room temperature for 2h. After completion, the mixture was concentrated under reduced pressure to get crude which was again dissolved in ethyl acetate and washed with 1N aq. sodium hydroxide solution. The organic part was dried over anhydrous sodium sulfate, filtered, and concentrated to give (2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-amine hydrochloride (**9**) as yellow viscous liquid. Yield: 0.94 g, 85%; LCMS m/z 476.2 $[M+H]^+$.

*Synthesis of methyl 2-(6-chloropyrazin-2-yl)-2,2-difluoroacetate (**11**)*

To a stirred suspension of copper (1.97 g, 3 eq., 31 mmol) in dimethyl sulfoxide (8 mL), methyl 2-bromo-2,2-difluoroacetate (**1a**, 3.7 mL, 3 eq., 31 mmol) was added at room temperature under inert atmosphere. After 30 minutes, 2-bromo-6-chloropyrazine (**10**, 2 g, 1 eq., 10.3 mmol) was added and heated at 90 °C for 3 h. After completion of reaction, the reaction mixture was quenched with saturated aqueous ammonium chloride solution and extracted with ethyl acetate (3 × 25 mL). The combined organic phase was washed with brine, dried with anhydrous sodium sulphate, filtered, and concentrated under reduced pressure to afford methyl 2-(6-chloropyrazin-2-yl)-2,2-difluoroacetate (2.5 g, crude) as dark brown liquid,

which was used directly in the next step. ^{1}H NMR (400 MHz, DMSO-d_6) for crude compound: δ 9.15 (s, 1H), 9.11 (s, 1H), 3.89 (s, 3H).

*Synthesis of 2-(6-chloropyrazin-2-yl)-2,2-difluoroethan-1-ol (**12**)*

To a stirred solution of methyl 2-(6-chloropyrazin-2-yl)-2,2-difluoroacetate (**11**, 2.5 g, crude) in methanol (20 mL), sodium borohydride (1.2 g, 3 eq., 33 mmol) was added portion wise for 10 min at 0 °C. After stirring 30 minutes at room temperature, reaction mixture was quenched in ice cold water and organic part was extracted with dichloromethane (3 × 20 mL). Combined dichloromethane layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude was purified by column chromatography using ethyl acetate/ heptane (0-30% gradient) as eluent to afford 2-(6-chloropyrazin-2-yl)-2,2-difluoroethan-1-ol as colourless liquid. Yield: 0.8 g, 39% over two steps. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.02 (s, 1H), 8.95 (s, 1H), 5.73 (t, J = 6.4 Hz, 1H), 4.03-3.94 (m, 2H).

*Synthesis of 2-(6-(((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluoroethan-1-ol (**13**)*

To a stirred solution of (2*R*,3*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2*H*-pyran-3-amine (**9**, 1.5 g, 1 eq., 3.15 mmol) and 2-(6-chloro-2-pyrazinyl)-2,2-difluoroethanol (**12**, 0.614 g, 1 eq., 3.15 mmol) in 1-methyl-2-pyrrolidinone (6 mL) was added *N,N*-Diisopropylethylamine (5.49 mL, 10 eq., 31.5 mmol). After that, the reaction mixture was heated at 200 °C for 48 h. After completion, reaction mixture was concentrated under reduced pressure to give crude which was again dissolved in ethyl acetate. Water (15 mL) was added and extracted with ethyl acetate (3 × 30 mL). The combined organic phase was washed with brine, dried over anhydrous sodium sulphate, filtered, and concentrated under reduced pressure to give crude which was purified by silica gel flash column chromatography using ethyl acetate/heptane as eluting system to afford 2-(6-(((2*R*,3*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2*H*-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluoroethan-1-ol (**13**) as brown viscous liquid. Yield: 0.95 g, 47%. LCMS *m/z* 634.3 $[M+H]^+$.

*Synthesis of tert-butyl (2-(2-(2-(2-(6-(((2R,3S,4R,5R,6R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluoroethoxy)ethoxy)ethoxy)ethyl)carbamate (**14**)*

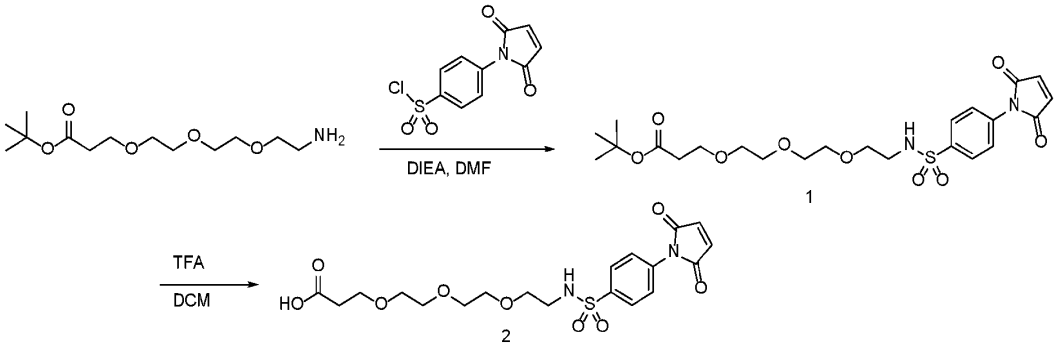
To a stirred solution of 2-(6-(((2*R*,3*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2*H*-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluoroethan-1-ol (**13**, 0.95 g, 1 eq., 1.35 mmol) in N, N-dimethylformamide (8 mL), sodium hydride (0.0594 g, 1.1 eq., 1.48 mmol) was added portion wise at 0 °C and stirred for 10 min. Then, *tert*-butyl (2-(2-(2-bromoethoxy)ethoxy)ethyl)carbamate (**2a**, 0.421 g, 1 eq., 1.35 mmol) was added and stirred at room

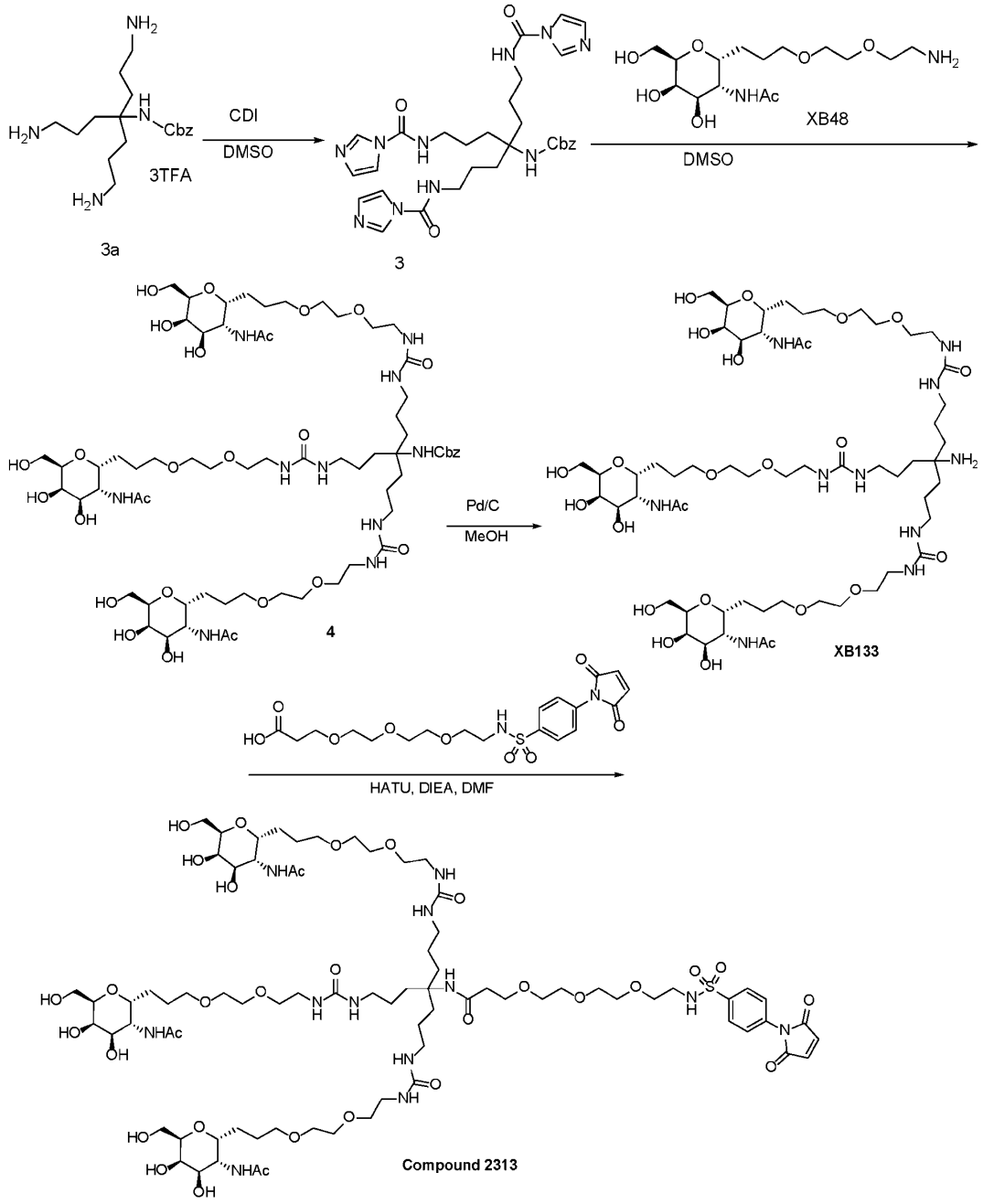
temperature for 3 h. After completion of the reaction, reaction mixture was quenched with ice-cold water and organic part was extracted with ethyl acetate (3 × 20 mL). Combined ethyl acetate layer was dried over anhydrous sodium sulphate, filtered, and concentrated under reduced pressure. The crude was purified by silica gel flash column chromatography using 40-50% ethyl acetate/ heptane to afford *tert*-butyl (2-(2-(2-(2-(6-(((2*R*,3*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2*H*-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluoroethoxy)ethoxy)ethoxy)ethyl)carbamate as light yellow viscous liquid. Yield: 0.9 g, 69.0%. LCMS: *m/z* 865.3 $[M+H]^+$.

Synthesis of (2R,3R,4R,5R,6R)-5-((6-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-1,1-difluoroethyl)pyrazin-2-yl)amino)-2-(hydroxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol ***(XB108)***

To a solution of *tert*-butyl (2-(2-(2-(2-(6-(((2*R*,3*S*,4*R*,5*R*,6*R*)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-propyltetrahydro-2*H*-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluoroethoxy)ethoxy)ethoxy)ethyl)carbamate (**14**, 0.96 g, 1 eq., 0.999 mmol) in dichloromethane (4 mL) was added boron trichloride (1.4 mL, 12 eq., 12 mmol, 1M solution) solution in dichloromethane dropwise at -78 °C and stirred at the same temperature for 3 h. After that, reaction mixture was quenched with methanol (2 mL) and solvent was evaporated under reduced pressure to afford crude product which was purified by reverse phase prep-HPLC (15-20% acetonitrile in water with 1% NH_4HCO_3). Fractions containing desired product were combined and lyophilized to afford (2*R*,3*R*,4*R*,5*R*,6*R*)-5-((6-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-1,1-difluoroethyl)pyrazin-2-yl)amino)-2-(hydroxymethyl)-6-propyltetrahydro-2*H*-pyran-3,4-diol (**XB108**) as white solid. Yield: 0.219 g, 40.0%. LCMS: *m/z* 495.3 $[M+H]^+$.

Synthesis of Compound 2313 from XB48





Synthesis of tert-butyl 3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propanoate **(1)**

To a mixture of 4-(2,5-dioxopyrrol-1-yl)benzenesulfonyl chloride (1.02 eq, 160 mg, 0.59 mmol) in DMF (1.6 mL) at 0 °C was added DIPEA (1.30 eq, 0.13 mL, 0.75 mmol) and a solution of tert-butyl 3-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)propanoate (1.00 eq, 161 mg, 0.58 mmol) in DMF (1.7 mL). The mixture was stirred at 0 °C for 1h then purified by prep HPLC (10 - 80% MeCN/water with 0.1% TFA) to give **1** as a clear syrup. Yield: 174.4 mg, 62%. LCMS m/z 457.0 $[M -Boc + H]^+$.

Synthesis of 3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propanoic acid (**2**)

To a mixture of tert-butyl 3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propanoate (**1**, 1.00 eq, 90.0 mg, 0.18 mmol) in DCM (1 mL) was added TFA (1 mL). The mixture was stirred at rt for 30 minutes then concentrated and purified by prep HPLC (5 - 70% MeCN/water) to give **2** as clear syrup. Yield: 66 mg, 82%. LCMS m/z 457.1 [M+H].

Synthesis of benzyl (4-(3-(1H-imidazole-1-carboxamido)propyl)-1,7-bis(1H-imidazole-1-carboxamido)heptan-4-yl)carbamate (**3**)

To a mixture of carbonyldiimidazole (7.00 eq, 162 mg, 1.00 mmol) in DMSO (0.5 mL) was added a solution of benzyl N-[4-amino-1,1-bis(3-aminopropyl)butyl]carbamate trifluoroacetate salt (**3a**, 1.00 eq, 97.0 mg, 0.14 mmol) in DMSO (1.3 mL) dropwise. The mixture was stirred at rt for 1h then diluted with EtOAc, washed with water (1x) and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with water (1x) and brine (1x), dried, concentrated, and purified by column (0 -10% MeOH/DCM) to give **3** as clear syrup. Yield: 62.7 mg, 71%. LCMS m/z 619.1 [M+H].

Synthesis of benzyl (1,31-bis((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-16-(15-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-5-oxo-9,12-dioxa-4,6-diazapentadecyl)-11,21-dioxo-4,7,25,28-tetraoxa-10,12,20,22-tetraazahentriacontan-16-yl)carbamate (**4**)

To a mixture of benzyl (4-(3-(1H-imidazole-1-carboxamido)propyl)-1,7-bis(1H-imidazole-1-carboxamido)heptan-4-yl)carbamate (**3**, 1.00 eq, 93.4 mg, 0.15 mmol) in DMSO (0.8 mL) was added a solution of N-[(2R,3R,4R,5R,6R)-2-[3-[2-(2-aminoethoxy)ethoxy]propyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (**XB48**, 3.00 eq, 159 mg, 0.45 mmol) in DMSO (2.2 mL). The mixture was stirred at 45 °C overnight then purified by prep HPLC (5-12-30% MeCN/water with 0.1% formic acid) to give **4** as a white solid. Yield: 129 mg, 58%. LCMS m/z 1465.7 [M+H].

Synthesis of N-[(2R,3R,4R,5R,6R)-2-[3-[2-[2-[[7-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylcarbamoylamino]-4-[3-[2-[2-[3-

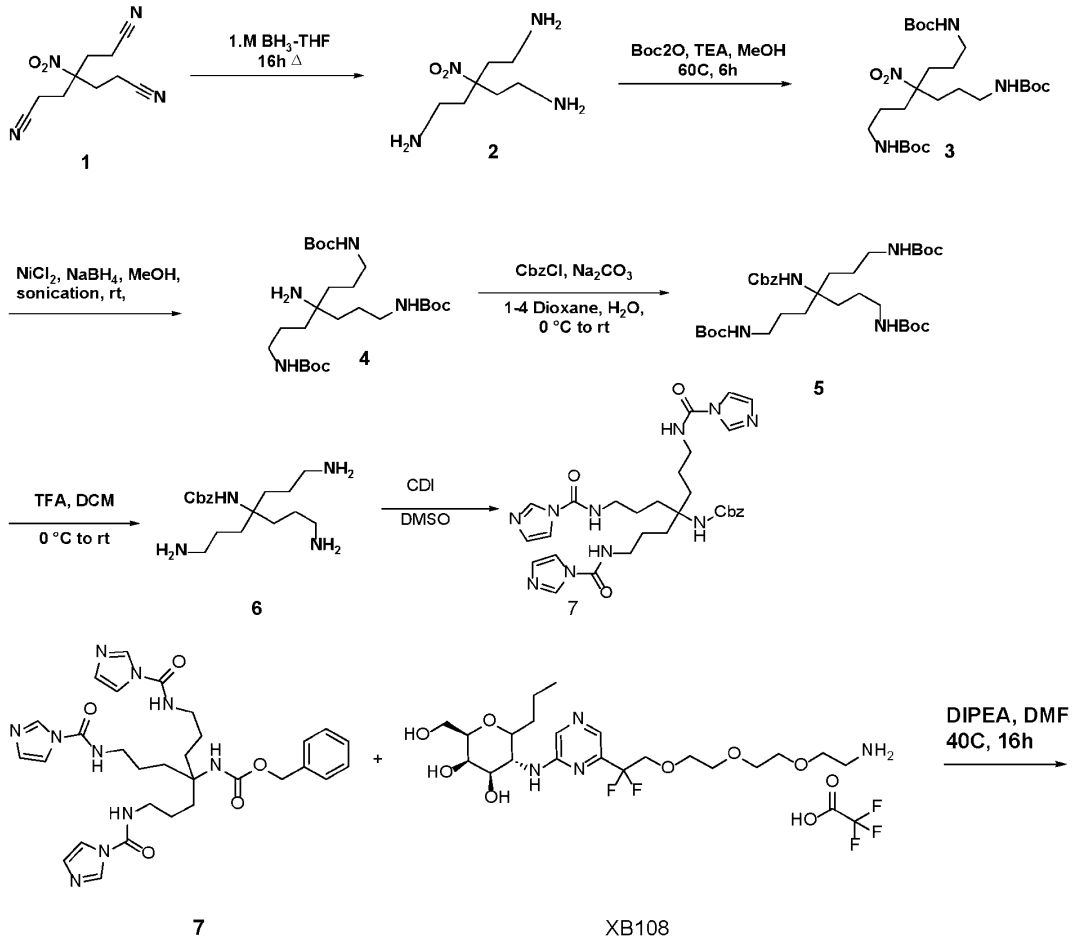
[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylcarbamoylamino]propyl]-4-amino-heptyl]carbamoylamino]ethoxy]ethoxy]propyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide **(XB133)**

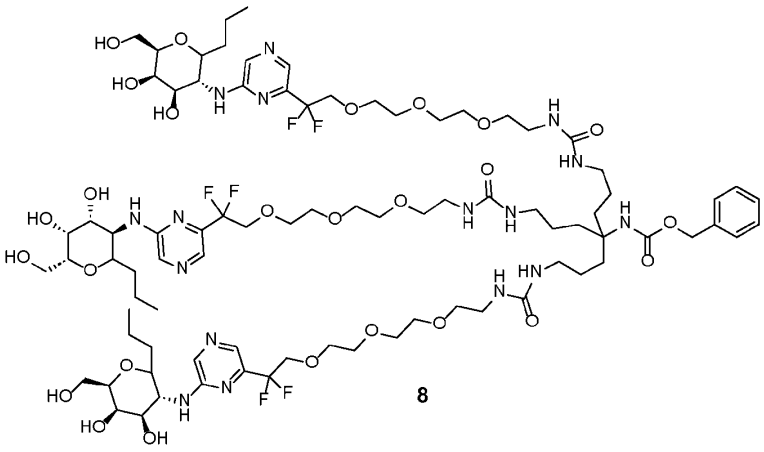
To a mixture of benzyl (1,31-bis((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-16-(15-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-5-oxo-9,12-dioxa-4,6-diazapentadecyl)-11,21-dioxo-4,7,25,28-tetraoxa-10,12,20,22-tetraazahentriacontan-16-yl)carbamate (**4**, 1.00 eq, 129 mg, 0.088 mmol) in MeOH (6 mL) was added 10% Pd/C (39 mg). The mixture was stirred at rt under hydrogen for 1h, filtered, concentrated, and purified by prep HPLC (3 - 50% MeCN/20 mM NH_4OH aqueous solution) to give **XB133** as a white solid. Yield: 104.2 mg, 89%. LCMS m/z 1331.7 [M+H].

Synthesis of ***Compound 2313***

To a mixture of 3-[2-[2-[2-[[4-(2,5-dioxopyrrol-1-yl)phenyl]sulfonylamino]ethoxy]ethoxy]ethoxy]propanoic acid (**2,** 1.10 eq, 3.7 mg, 0.0082 mmol) in DMF (0.25 mL) were added DIEA (3.00 eq, 0.0039 mL, 0.022 mmol) , HATU (1.10 eq, 3.1 mg, 0.0082 mmol) and N-[(2R,3R,4R,5R,6R)-2-[3-[2-[2-[[7-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylcarbamoylamino]-4-[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylcarbamoylamino]propyl]-4-amino-heptyl]carbamoylamino]ethoxy]ethoxy]propyl]-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]acetamide (**Compound 2313,** 1.00 eq, 9.9 mg, 0.0074 mmol) . The mixture was stirred at rt for 30 minutes. To a solution of 3-[2-[2-[2-[[4-(2,5-dioxopyrrol-1-yl)phenyl]sulfonylamino]ethoxy]ethoxy]ethoxy]propanoic acid (0.68 mg) in DMF (0.1 mL) were added DIEA (1 uL) and HATU (0.6 mg). This mixture was added to the above reaction mixture. The mixture was stirred at rt for 30 minutes and purified by prep. HPLC (5 - 13 -25% MeCN/water with 0.1% TFA) to give **Compound 2313** as a white solid. Yield: 8.2 mg, 62%. LCMS m/z 1771.1 [M+H].

Synthesis of N-(1,35-bis(6-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-18-(1-(6-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)-1,1,35,35-tetrafluoro-13,23-dioxo-3,6,9,27,30,33-hexaoxa-12,14,22,24-tetraazapentatriacontan-18-yl)-3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propanamide (2301) from XB108:





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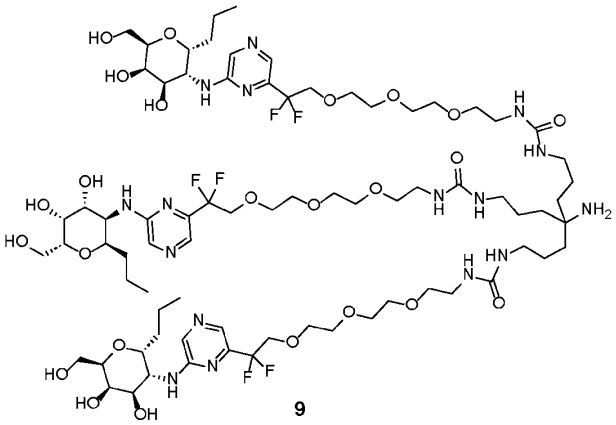
Pd/H_2
Methanol, 1h



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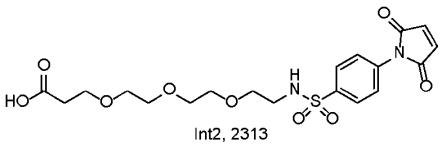
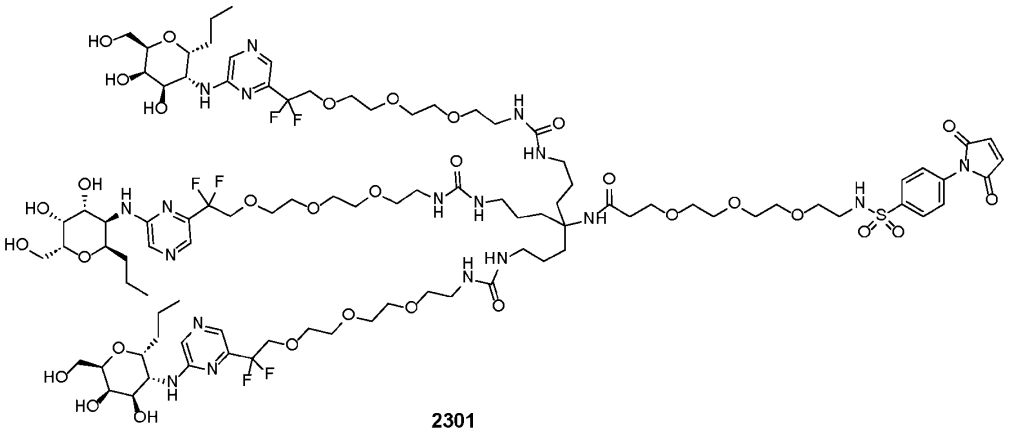


Int2, 2313

HATU, DIPEA, DMF, 1h



2301

Synthesis of 4-(3-aminopropyl)-4-nitroheptane-1,7-diamine, (**2**)

A mixture of 4-(2-cyanoethyl)-4-nitro-heptanedinitrile (**1**, 1.00 eq, 1.00 g, 4.54 mmol) in THF (3 mL) was treated with a solution of 2M DMS-borane in THF (5.00 eq, 11 mL, 22.7 mmol) and the reaction was heated to reflux (90C external) for 18 h. The reaction was monitored by LCMS. After cooling to rt, concentrated hydrogen chloride (10.0 eq, 3.8 mL, 45.4 mmol) was added by an addition funnel over 10 minutes to quench excess borane then the mixture was heated in a steam bath for 30 minutes. All solvent was removed under vacuum and the residue was basified with 40% NaOH solution (6 mL) and brine (6mL) then extracted with CH2Cl2 (20 mL×6) The organic phase was dried over Na2SO4, filtered and concentrated under vacuum and the crude residue (**2**) was used directly in the next. LCMS: m/z 233.01 [M+H]+

Synthesis of di-tert-butyl (4-(3-((tert-butoxycarbonyl)amino)propyl)-4-nitroheptane-1,7-diyl)dicarbamate **(3)**

A solution of 4-(3-aminopropyl)-4-nitro-heptane-1,7-diamine (**2**, 1.00 eq, 1.05 g, 4.52 mmol) was dissolved in 12 volumes of methanol (12 mL) before adding triethylamine (4.00 eq, 2.5 mL, 18.1 mmol) followed by di-tert-butyl dicarbonate (3.30 eq, 3255 mg, 14.9 mmol) and the reaction was heated to 60C for 6h. Volatile components were removed under reduced pressure and the residue was dissolved in DCM then adsorbed to silica and purified by silica gel chromatography (25-50%% EtOAc in hexanes) to give (**3**) tert-butyl N-[7-(tert-butoxycarbonylamino)-4-[3-(tert-butoxycarbonylamino)propyl]-4-nitro-heptyl]carbamate (**3**, 1.53 g, 2.86 mmol, 63.35 % yield over 2 steps). LCMS: m/z 532.7 $[M+H]^+$

Synthesis of di-tert-butyl (4-amino-4-(3-((tert-butoxycarbonyl)amino)propyl)heptane-1,7-diyl)dicarbamate **(4)**

A solution of Nickel(II) Chloride hexahydrate (0.601 eq, 67.0 mg, 0.282 mmol) in methanol (3 mL) was treated with sodium borohydride (1.63 eq, 29.0 mg, 0.767 mmol) and the slurry was sonicated for 30 minutes. Next, a solution of tert-butyl N-[7-(tert-butoxycarbonylamino)-4-[3-(tert-butoxycarbonylamino)propyl]-4-nitro-heptyl]carbamate (**3**, 1.00 eq, 250 mg, 0.469 mmol) in methanol (3 mL) was added followed by additional sodium borohydride (57 mg, 1.5 mmol) and the bubbling slurry was sonicated for 30 minutes. Additional sodium borohydride (57 mg, 1.5 mmol) was added a second time. After another 30 minutes of sonication, celite was added to the slurry and the mixture was filtered through a short pad of celite and the solvent was evaporated. The residue oil was partitioned between 20 mL water and 20mL DCM. The aqueous layer was washed with DCM (2x10mL) and the combined organic extracts dried over Na2SO4, filtered and concentrated under reduced pressure to give 169 mg

crude di-tert-butyl (4-amino-4-(3-((tert-butoxycarbonyl)amino)propyl)heptane-1,7-diyl)dicarbamate (**4**) (72% yield). LCMS: m/z 503.2 $[M+H]^+$

Synthesis of benzyl di-tert-butyl (4-(3-((tert-butoxycarbonyl)amino)propyl)heptane-1,4,7-triyl)tricarbamate (**5**)

A solution of di-tert-butyl (4-amino-4-(3-((tert-butoxycarbonyl)amino)propyl)heptane-1,7-diyl)dicarbamate (**4,** 1.0 eq, 1.1 g, 2.19 mmol) in 1,4-dioxane (9 mL) and water (3 mL) was cooled at 0 °C, sodium carbonate (3.0 eq, 0.70 g, 6.56 mmol) and benzyl chloroformate (50 % solution in toluene) (1.5 eq, 1.12 mL, 3.28 mmol) were added and reaction mixture was stirred at room temperature for 16 h. After completion, water was added to the reaction mixture and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh, 0-15 % ethyl acetate in hexane) to afford benzyl di-tert-butyl (4-(3-((tert-butoxycarbonyl)amino)propyl)heptane-1,4,7-triyl)tricarbamate (**5**) as a colorless viscous liquid. Yield: 1.3 g, 93.3 %; LCMS m/z 637.55 $[M+1]^+$.

Synthesis of benzyl (1,7-diamino-4-(3-aminopropyl)heptan-4-yl)carbamate (**6**)

A solution of benzyl di-tert-butyl (4-(3-((tert-butoxycarbonyl)amino)propyl)heptane-1,4,7-triyl)tricarbamate (**5**, 1.0 eq, 1.3 g, 2.04 mmol) in dichloromethane (13 mL) was cooled at 0 °C, trifluoroacetic acid (13 mL) was added and reaction mixture was stirred at room temperature for 1 h. After completion, reaction mixture was concentrated, azeotroped with dichloromethane (2-3 times), dissolved in minimum amount of water, neutralized with ammonia solution (pH~8) and concentrated to get crude which was purified by prep HPLC (15-25 % acetonitrile in water with 0.1 % ammonium hydroxide) to afford **6** as an off white solid. Yield: 0.495 g, 72.1 %; LCMS m/z 337.20 $[M+1]^+$; 1H NMR (400 MHz, DMSO-d_6 with D_2O & d-TFA) δ 7.34-7.26 (m, 5H), 4.96 (s, 2H), 2.72 (t, J = 6.8 Hz, 6H), 1.62-1.52 (m, 6H), 1.51-1.41 (m, 6H).

Synthesis of benzyl (4-(3-(1H-imidazole-1-carboxamido)propyl)-1,7-bis(1H-imidazole-1-carboxamido)heptan-4-yl)carbamate (**7**)

To a mixture of carbonyldiimidazole (7.00 eq, 162 mg, 1.00 mmol) in DMSO (0.5 mL) was added a solution of benzyl N-[4-amino-1,1-bis(3-aminopropyl)butyl]carbamate trifluoroacetate salt (**6**, 1.00 eq, 97.0 mg, 0.14 mmol) in DMSO (1.3 mL) dropwise. The mixture was stirred at rt for 1h then diluted with EtOAc, washed with water (1x) and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with water (1x) and brine (1x), dried, concentrated, and purified by column (0 -10% MeOH/DCM) to give **7** as clear syrup. Yield: 62.7 mg, 71%. LCMS m/z 619.1 [M+H].

Synthesis of benzyl (1,35-bis(6-(((3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-18-(1-(6-(((3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)-1,1,35,35-tetrafluoro-13,23-dioxo-3,6,9,27,30,33-hexaoxa-12,14,22,24-tetraazapentatriacontan-18-yl)carbamate (**8**)

A solution of benzyl N-[4-(imidazole-1-carbonylamino)-1,1-bis[3-(imidazole-1-carbonylamino)propyl]butyl]carbamate (**7**, 1.00 eq, 8.0 mg, 0.0129 mmol) in DMF (90 uL) was added to a basic solution of (2R,3R,4R,5R)-5-[[6-[2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]-1,1-difluoro-ethyl]pyrazin-2-yl]amino]-2-(hydroxymethyl)-6-propyl-tetrahydropyran-3,4-diol;2,2,2-trifluoroacetic acid (**XB108**, 3.30 eq, 26.0 mg, 0.0427 mmol) and N,N-diisopropylethylamine (DIPEA) (3.00 eq, 6.8 uL, 0.0388 mmol) in DMF (90 uL). The reaction was heated to 40 C overnight. The reaction was diluted with DMSO to 800 uL then purified by reversed-phase HPLC (5-50% acetonitrile in water w/0.2 mM NH4OH) to give product **8** (23 mg, 0.0121 mmol, 93.71 % yield). LCMS: m/z 1898.4 [M+H]+

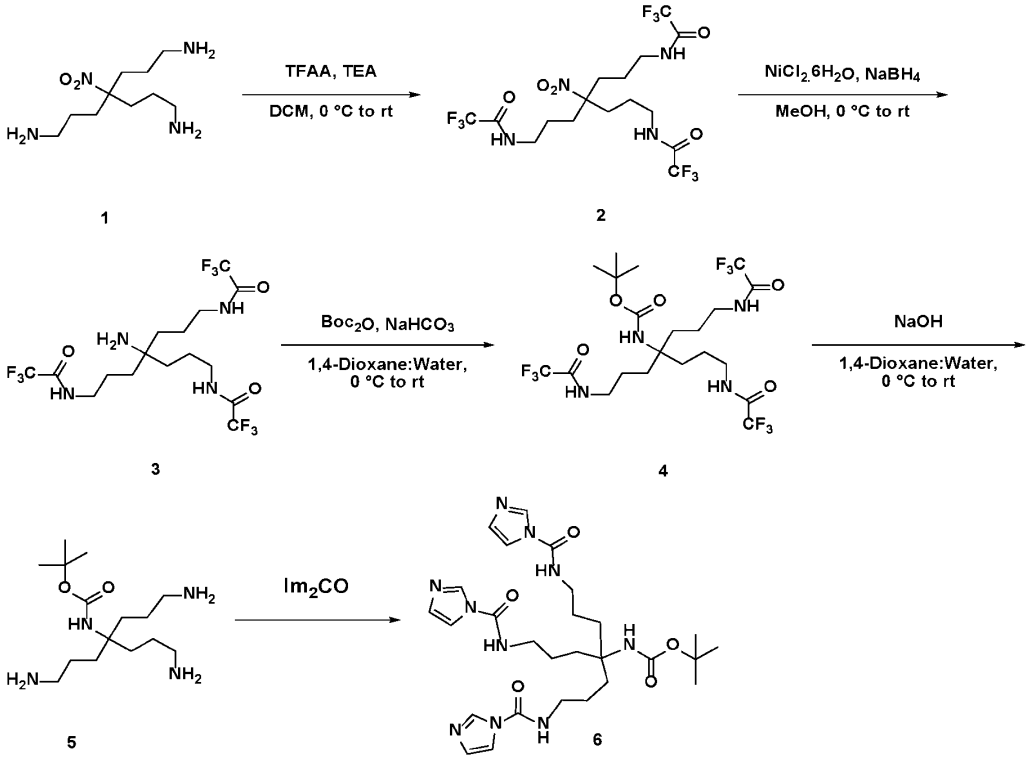
Synthesis of 1,1'-(4-amino-4-(1-(6-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)heptane-1,7-diyl)bis(3-(2-(2-(2-(2-(6-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluoroethoxy)ethoxy)ethoxy)ethyl)urea) (**9**)

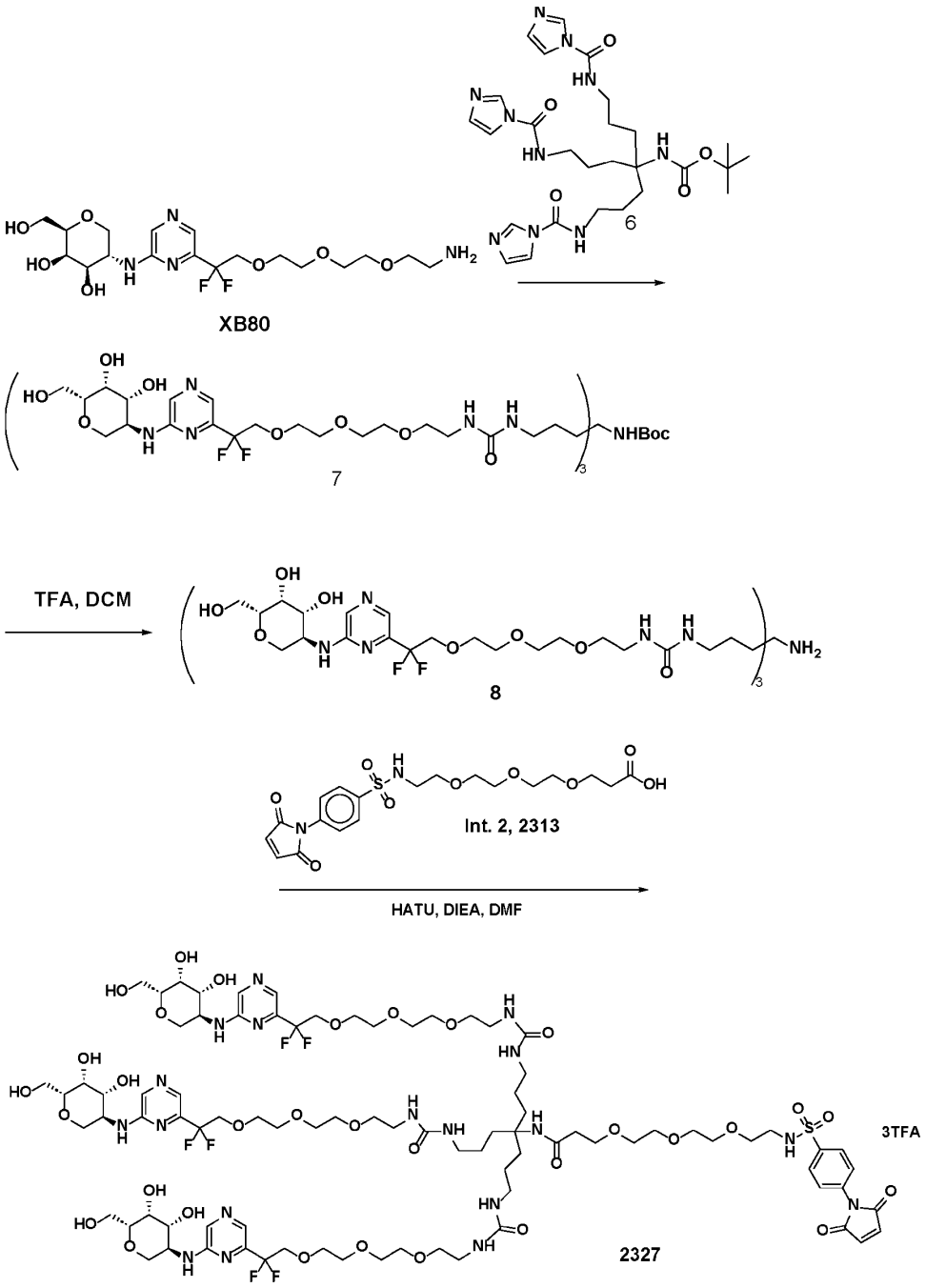
A solution of **8** (1.00 eq, 23.0 mg, 0.012 mmol) in methanol (0.250 mL) and a drop of acetic acid was purged with nitrogen before being treated with 10% Pd/C (0.500 eq, 12.9 mg, 0.00606 mmol) then the vial was evacuated then back-filled with hydrogen via balloon, a process that was repeated 2 more times before leaving under an atmosphere of hydrogen for 1hr. The reaction was filtered (through 0.45um syringe filter), concentrated under reduced pressure then dissolved in DMSO and purified by reversed-phase HPLC (5-50% MeCN in water w/0.2 mM NH4OH) to give product **9** (16 mg, 0.0090 mmol, 74.8 % yield). LCMS: m/z 1763.4 [M+H]+

Synthesis of N-(1,35-bis(6-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-18-(1-(6-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)-1,1,35,35-tetrafluoro-13,23-dioxo-3,6,9,27,30,33-hexaoxa-12,14,22,24-tetraazapentatriacontan-18-yl)-3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propanamide (**2301**)

A solution of 3-[2-[2-[2-[[4-(2,5-dioxopyrrol-1-yl)phenyl]sulfonylamino]ethoxy]ethoxy]ethoxy]propanoic acid (1.45 eq, 6.1 mg, 0.0133 mmol) and HATU (1.60 eq, 5.6 mg, 0.0147 mmol) was treated with N,N-diisopropylethylamine (7.00 eq, 11 uL, 0.0641 mmol) for 1 minute before adding to a solution of **9** (1.00 eq, 16.2 mg, 0.00916 mmol) in DMF (125 uL). After 2h, the reaction was treated with trifluoroacetic acid (7.00 eq, 0.0049 mL, 0.0641 mmol) and diluted with water, then purified by reversed-phase HPLC (C18, eluting with 5-50% acetonitrile in water with 0.1% TFA) to give product (6.0 mg, 0.00272 mmol, 29.7 % yield) as a white solid. LCMS: m/z 1102.2 $[M+2H]^{++}$

Synthesis of N-(1,35-bis(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-18-(1-(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)-1,1,35,35-tetrafluoro-13,23-dioxo-3,6,9,27,30,33-hexaoxa-12,14,22,24-tetraazapentatriacontan-18-yl)-3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propenamide (2327):





Synthesis of N,N'-(4-nitro-4-(3-(2,2,2-trifluoroacetamido)propyl)heptane-1,7-diyl)bis(2,2,2-trifluoroacetamide) (**2**)

A solution of 4-(3-aminopropyl)-4-nitroheptane-1,7-diamine (**1**, 1.0 eq, 2.0 g, 8.61 mmol) in dichloromethane (40 mL) was cooled at 0 °C, triethylamine (10.0 eq, 12.4 mL, 86.1 mmol) and trifluoroacetic anhydride (TFAA) (6.0 eq, 7.75 mL, 51.7 mmol) were added and reaction mixture was stirred at room temperature for 16 h. After completion, reaction mixture was concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-40 % ethyl acetate in hexane to afford N,N'-(4-nitro-4-(3-(2,2,2-trifluoroacetamido)propyl)heptane-1,7-diyl)bis(2,2,2-trifluoroacetamide) (**2**) as a colorless viscous liquid. Yield: 2.5 g, 55.8 %; LCMS m/z 518.8 $[M-1]^-$.

Synthesis of N,N'-(4-amino-4-(3-(2,2,2-trifluoroacetamido)propyl)heptane-1,7-diyl)bis(2,2,2-trifluoroacetamide) (**3**)

A solution of N,N'-(4-nitro-4-(3-(2,2,2-trifluoroacetamido)propyl)heptane-1,7-diyl)bis(2,2,2-trifluoroacetamide) (**2**, 1.0 eq, 0.500 g, 0.961 mmol) in methanol (5 mL) was cooled at 0 °C, nickel chloride hexahydrate (1.0 eq, 0.228 g, 0.961 mmol) and sodium borohydride (6.0 eq, 0.218 g, 5.77 mmol) were added and reaction mixture was stirred at room temperature for 2 h. After completion, the reaction mixture was filtered through celite bed and celite bed was washed with methanol. The filtrate was concentrated, water was added and extracted with 10 % methanol in dichloromethane. The organic layer was dried over anhydrous sodium sulfate, filtered, concentrated and dried to afford N,N'-(4-amino-4-(3-(2,2,2-trifluoroacetamido)propyl)heptane-1,7-diyl)bis(2,2,2-trifluoroacetamide) (**3**) as a colorless viscous liquid. Yield: 0.390 g, 82.7 %; LCMS m/z 491.3 $[M+1]^+$.

Synthesis of tert-butyl (1,7-bis(2,2,2-trifluoroacetamido)-4-(3-(2,2,2-trifluoroacetamido)propyl)heptan-4-yl)carbamate (**4**)

A solution of N,N'-(4-amino-4-(3-(2,2,2-trifluoroacetamido)propyl)heptane-1,7-diyl)bis(2,2,2-trifluoroacetamide) (**3**, 1.0 eq, 0.840 g, 1.71 mmol) in 1,4-dioxane (12.8 mL) and water (3.2 mL) was cooled at 0 °C, sodium bicarbonate (3.0 eq, 0.432, 5.14 mmol) and boc anhydride (1.1 eq, 0.45 mL, 1.88 mmol) were added and reaction mixture was stirred at room temperature for 16 h. Reaction was monitored by TLC & ELSD. After completion, reaction mixture was concentrated to get crude which was purified by column chromatography using silica gel (100-200 mesh) and 0-40 % ethyl acetate in hexane to afford tert-butyl (1,7-bis(2,2,2-trifluoroacetamido)-4-(3-(2,2,2-trifluoroacetamido)propyl)heptan-4-yl)carbamate (**4**) as a white solid. Yield: 0.820 g, 81.0 %; LCMS m/z 588.8 $[M-1]^-$.

Synthesis of tert-butyl (1,7-diamino-4-(3-aminopropyl)heptan-4-yl)carbamate (**5**)

A solution of tert-butyl (1,7-bis(2,2,2-trifluoroacetamido)-4-(3-(2,2,2-trifluoroacetamido)propyl)heptan-4-yl)carbamate (**4**, 1.0 eq, 0.940 g, 1.59 mmol) in 1,4-dioxane (5 mL) was cooled at 0 °C, a solution of sodium hydroxide (3.0 eq, 0.190 g, 4.77 mmol) in water (5 mL) was

added and reaction mixture was stirred at room temperature for 16 h. After completion, the reaction mixture was concentrated and dried to get crude which was purified by prep HPLC (15-30 % acetonitrile in water with 0.1 % ammonium hydroxide). Fractions containing the desired product were combined and lyophilized to afford tert-butyl (1,7-diamino-4-(3-aminopropyl)heptan-4-yl)carbamate (**5**) as a white solid. Yield: 0.252 g, 52.6 %; LCMS m/z 303.2 $[M+1]^+$; 1H NMR (400 MHz, DMSO-d_6 with D_2O) δ 2.85-2.72 (m, 2H), 2.65-2.52 (m, 4H), 1.58-1.38 (m, 6H), 1.36-1.18 (m, 15H).

Synthesis of tert-butyl (4-(3-(1H-imidazole-1-carboxamido)propyl)-1,7-bis(1H-imidazole-1-carboxamido)heptan-4-yl)carbamate (**6**)

A solution of carbonyldiimidazole (8.00 eq, 6.43 g, 39.7 mmol) in DMSO (17 mL) was stirred for 5 minutes before adding solid tert-butyl N-[4-amino-1,1-bis(3-aminopropyl)butyl]carbamate (**5**, 1.00 eq, 1.50 g, 4.96 mmol) followed by DMSO (5 mL) for rinsing. Solids dissolved within 5 minutes. After 1h, the reaction was injected directly to prep-HPLC for purification (5-50% MeCN in water) to give tert-butyl N-[4-(imidazole-1-carbonylamino)-1,1-bis[3-(imidazole-1-carbonylamino)propyl]butyl]carbamate (**6**, 1.54 g, 2.63 mmol, 53.11 % yield). LCMS: m/z 585.4 [M+H]+

Synthesis of tert-butyl (1,35-bis(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-18-(1-(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)-1,1,35,35-tetrafluoro-13,23-dioxo-3,6,9,27,30,33-hexaoxa-12,14,22,24-tetraazapentatriacontan-18-yl)carbamate (**7**)

To a mixture of (2R,3R,4R,5S)-5-[[6-[2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]-1,1-difluoro-ethyl]pyrazin-2-yl]amino]-2-(hydroxymethyl)tetrahydropyran-3,4-diol (3.16 eq, 120 mg, 0.265 mmol) in DMSO (0.5 mL) was added a solution of tert-butyl N-[4-(imidazole-1-carbonylamino)-1,1-bis[3-(imidazole-1-carbonylamino)propyl]butyl]carbamate (**6**, 1.00 eq, 49.1 mg, 0.0840 mmol) in DMSO (0.9 mL). The mixture was stirred at 40 °C for 3h and at room overnight, purified by preparative HPLC (C18, eluting with 5 - 15 - 40%MeCN/water with 0.1% TFA) to give **7** as a white solid (145 mg, yield: 83%). LCMS m/z 1737.5 [M + H]+

Synthesis of 1,1'-(4-amino-4-(1-(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)heptane-1,7-diyl)bis(3-(2-(2-(2-(2-(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-2,2-difluorocthoxy)cthoxy)cthoxy)cthyl)urca) (**8**)

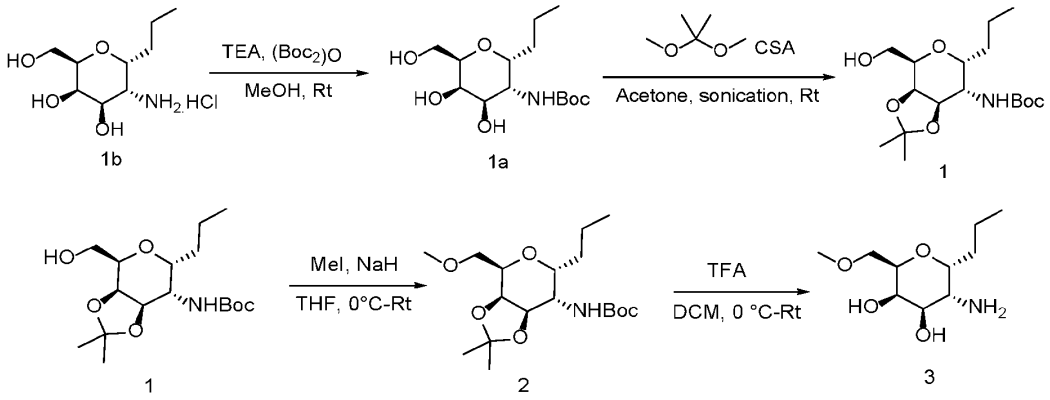
To a mixture of tert-butyl N-[4-[2-[2-[2-[2,2-difluoro-2-[6-[[rac-(3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]amino]pyrazin-2-yl]ethoxy]ethoxy]ethoxy]ethylcarbamoylamino]-

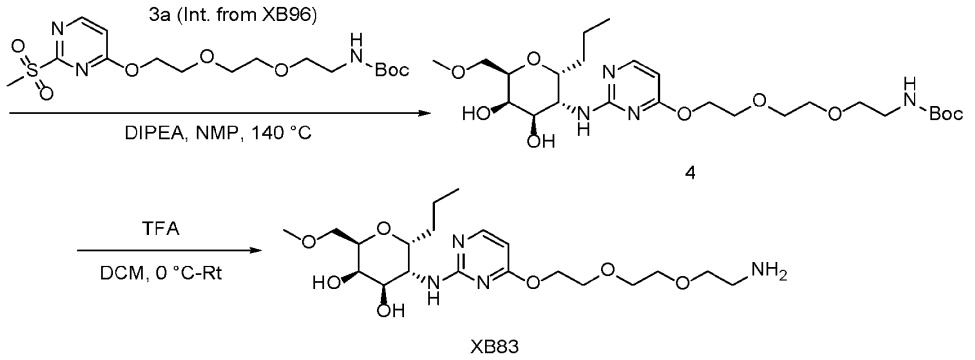
1,1-bis[3-[2-[2-[2-[2,2-difluoro-2-[6-[[rac-(3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-3-yl]amino]pyrazin-2-yl]ethoxy]ethoxy]ethoxy]ethylcarbamoylamino]propyl]butyl]carbamate;2,2,2-trifluoroacetic acid (**7**, 1.00 eq, 145 mg, 0.0697 mmol) in DCM (1 mL) was added TFA (1 mL). The mixture was stirred at rt for 1h, concentrated, and purified by prep. HPLC (5 - 70% MeCN/20 mM NH4OH solution) to give **8** as a white solid (114.2 mg, yield: 100%). LCMS 1637.5 [M + H]+

N-(1,35-bis(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-18-(1-(6-(((3S,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)amino)pyrazin-2-yl)-1,1-difluoro-13-oxo-3,6,9-trioxa-12,14-diazaheptadecan-17-yl)-1,1,35,35-tetrafluoro-13,23-dioxo-3,6,9,27,30,33-hexaoxa-12,14,22,24-tetraazapentatriacontan-18-yl)-3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propenamide (**2327**)

To a mixture of 3-[2-[2-[2-[[4-(2,5-dioxopyrrol-1-yl)phenyl]sulfonylamino]ethoxy]ethoxy]ethoxy]propanoic acid (Intermediate 2 from 2313 synthesis, 1.40 eq, 9.9 mg, 0.0216 mmol) in DMF (0.3 mL) at room temperature were added diisopropylethylamine (3.00 eq, 0.0081 mL, 0.0463 mmol) and hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU, 1.40 eq, 8.2 mg, 0.0216 mmol) , and this reaction mixture was added to a solution of **8** (1.00 eq, 25.3 mg, 0.0154 mmol) in DMF (0.2 mL). The mixture was stirred at rt for 2h and purified by prep. HPLC (5 - 20 -45% MeCN/water with 0.1% TFA) to give **2327** as a white solid, TFA salt (24 mg, yield: 63.7%). LCMS: 1039.3 $[M + 2H]^{++}$.

Synthesis of (2R,3R,4R,5R,6R)-5-((4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)pyrimidin-2-yl)amino)-2-(methoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol (XB83)





Tert-butyl ((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**1a**)

To a stirred solution of (2R,3R,4R,5R,6R)-5-amino-2-(hydroxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol hydrochloride (**1b**, 5.3 g, 21.9 mmol) in methanol (45 mL), were added triethylamine (3 eq., 9 mL, 65.8 mmol) and di-tert-butyl dicarbonate (1.2 eq., 6.04 mL, 26.3 mmol) gradually at 0° C under nitrogen atmosphere. Then reaction mixture was stirred at room temperature for 12h. After completion the reaction mixture was concentrated under reduced pressure to get a crude which was diluted with water and extracted with ethyl acetate (5 X 120 mL). Organic part was dried over anhydrous sodium sulphate, filtered and concentrated under reduced pressure to afford tert-butyl ((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**1a**) as off white solid. Yield: 5.0 g, crude; LCMS m/z 306.1$[M + H]^+$.

tert-butyl ((3aR,4R,6R,7S,7aR)-4-(hydroxymethyl)-2,2-dimethyl-6-propyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)carbamate (**1**)

To a stirred solution of tert-butyl ((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)carbamate (**1a**, 1.0 eq., 3.0 g, 9.82 mmol) in 2,2-dimethoxypropane (15.0 mL, 22 eq., 218 mmol) and acetone (15.0 mL) was added camphor sulfonic acid (0.2 eq., 0.454 g, 1.96 mmol) at 0°C and reaction mixture was sonicated for 30 min. Then 0.5 mL of methanol was added to the reaction mixture and reaction was stirred at room temperature for 10 min. After completion of reaction, reaction mixture was neutralized using triethyl amine and concentrated under reduced pressure to afford crude which was purified by silica gel flash column chromatography using 20-50 % ethyl acetate/hexane as eluent to afford tert-butyl ((3aR,4R,6R,7S,7aR)-4-(hydroxymethyl)-2,2-dimethyl-6-propyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)carbamate (**1**) as off white solid. Yield: 1.6 g, 47%; ELSD-MS m/z 346.2 $[M + H]^+$.

Synthesis of tert-butyl ((3aR,4R,6R,7S,7aR)-4-(methoxymethyl)-2,2-dimethyl-6-propyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)carbamate (**2**)

To a stirred solution of tert-butyl ((3aR,4R,6R,7S,7aR)-4-(hydroxymethyl)-2,2-dimethyl-6-propyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)carbamate (**1,** 1.0 g, 1 eq., 2.89 mmol) in dry tetrahydrofuran (15 mL), sodium hydride (127 mg, 1.1 eq., 3.18 mmol) was added portion-wise at 0 °C under nitrogen atmosphere. The reaction was allowed to stir at room temperature for 30 min. After that, methyl iodide (0.360 mL, 2 eq., 5.79 mmol) was added slowly at 0 °C, and stirred at room temperature for another 30 min. After completion (monitored by TLC), ice-cold water was added to the reaction mixture and extracted with dichloromethane (3 × 10 mL). Combined organic portions were dried over anhydrous sodium sulphate and concentrated under reduced pressure to give crude residue which was purified by silica gel flash column chromatography using 40% ethyl acetate in heptane as the eluent to afford tert-butyl ((3aR,4R,6R,7S,7aR)-4-(methoxymethyl)-2,2-dimethyl-6-propyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)carbamate (**2**) as yellow sticky liquid. Yield**:** 0.75 g, 72.0%. LCMS m/z 360.20 [M+H].

Synthesis of (2R,3R,4R,5R,6R)-5-amino-2-(methoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol (**3**)

To a stirred solution of ((3aR,4R,6R,7S,7aR)-4-(methoxymethyl)-2,2-dimethyl-6-propyltetrahydro-4H-[1,3]dioxolo[4,5-c]pyran-7-yl)carbamate (**2,** 1.1 g, 1.0 eq., 3.06 mmol) in dichloromethane (15 mL), trifluoroacetic acid (15 mL) was added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 4h. After completion (monitored by TLC), reaction mixture was concentrated under reduce pressure and co-evaporated with dichloromethane three times to obtain crude residue, which was further lyophilized to afford (2R,3R,4R,5R,6R)-5-amino-2-(methoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol (**3**) as light yellow syrup. The crude residue was directly used for next step. Yield: 0.6 g (Crude). LCMS m/z 220.1 $[M+H]^+$.

Synthesis of tert-butyl (2-(2-(2-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)ethoxy)ethoxy)ethyl)carbamate (**4**)

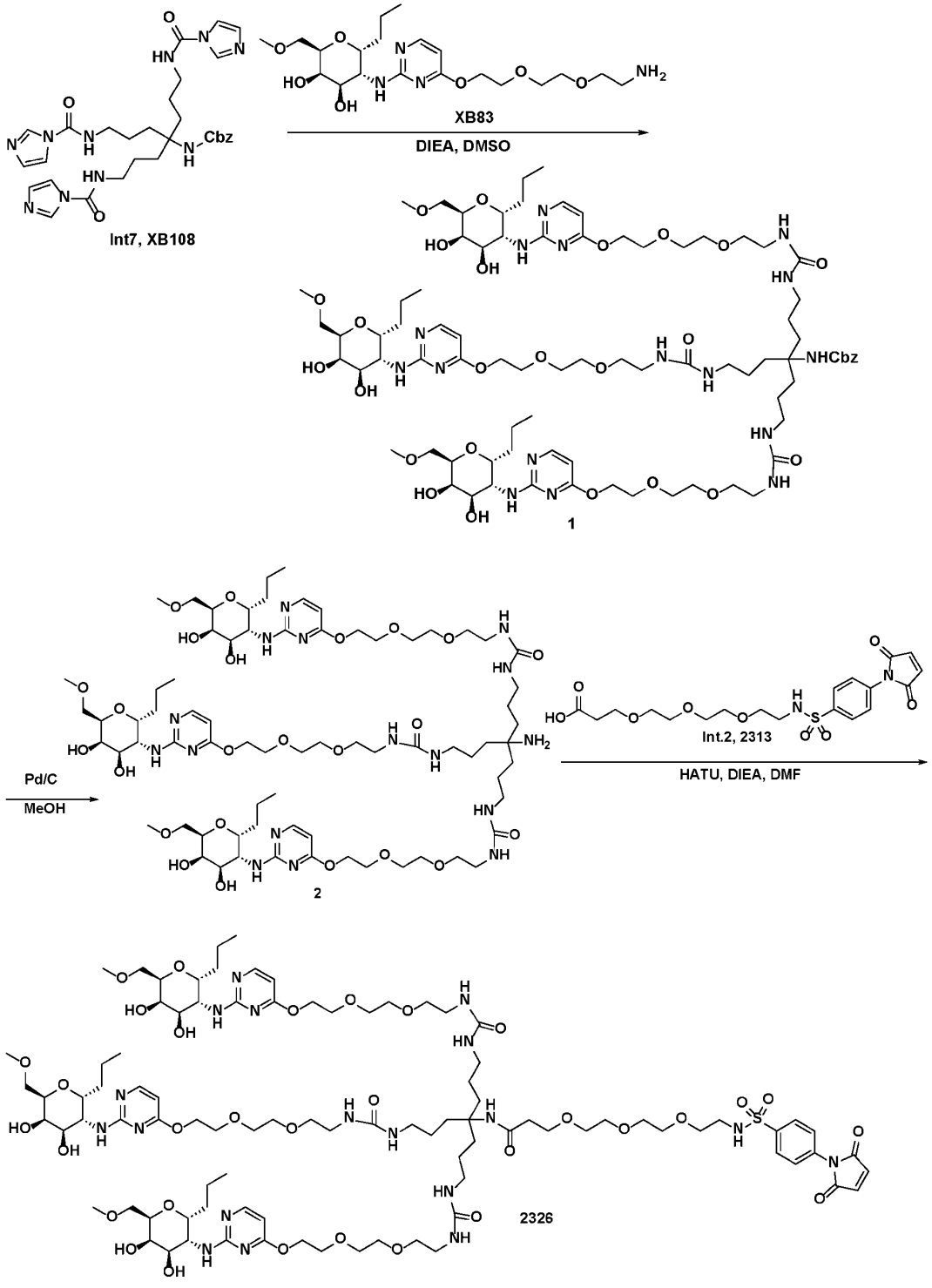
To a stirred solution of (2R,3R,4R,5R,6R)-5-amino-2-(methoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol (**3,** 0.7 g, 1.2 eq., 2.1 mmol) in dry 1-methyl-2-pyrrolidinone (2 mL), N,N-Diisopropylethylamine (3.66 mL, 12 eq., 21 mmol) was added at room temperature and then heated at 140 °C for 16h. After completion (monitored by ELSD-MS), reaction mixture was concentrated under reduced pressure. The crude residue was purified by silica gel flash column chromatography using 2-3% methanol in dichloromethane as the eluent to afford tert-butyl (2-(2-(2-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-

yl)oxy)ethoxy)ethoxy)ethyl)carbamate (**4**) as brown gummy liquid. Yield: 0.64 g, 67.1%. LCMS m/z 545.90 $[M+H]^+$.

Synthesis of (2R,3R,4R,5R,6R)-5-((4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)pyrimidin-2-yl)amino)-2-(methoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol (**XB83**)

A solution of tert-butyl (2-(2-(2-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)ethoxy)ethoxy)ethyl)carbamate (**4,** 0.140 g, 1 eq., 0.257 mmol) in dichloromethane (1 mL) was cooled at 0 °C and trifluoroacetic acid (1 mL) was added and the reaction mixture was stirred at room temperature for 3h. After completion, the reaction mixture was concentrated under reduce pressure to get crude residue which was purified by RP prep HPLC (25% acetonitrile in water with 0.1% TFA) to afford ((2R,3R,4R,5R,6R)-5-((4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)pyrimidin-2-yl)amino)-2-(methoxymethyl)-6-propyltetrahydro-2H-pyran-3,4-diol (**XB83**) as colorless sticky solid. Yield: 0.058 g, 50.8%. LCMS m/z 445.3 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-D_6 with D_2O): δ 7.99 (d, J = 6.4 Hz, 1H), 6.18 (d, J = 6.0 Hz, 1H), 4.44-4.43 (m, 2H), 4.18-4.14 (m, 1H), 4.10-4.06 (m, 1H), 3.75-3.70 (m, 5H), 3.60-3.58 (m, 6H), 3.50 (d, J = 4.4 Hz, 1H), 3.25 (s, 3H), 2.97 (t, J = 5.2 Hz, 2H), 1.61-1.55 (m, 1H), 1.33-1.18 (m, 4H), 0.81 (t, J = 6.8 Hz, 3H).

Synthesis of N-(1,29-bis((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-15-(1-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-10-oxo-3,6-dioxa-9,11-diazatetradecan-14-yl)-10,20-dioxo-3,6,24,27-tetraoxa-9,11,19,21-tetraazanonacosan-15-yl)-3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propanamide (2326):


Int7, XB108
XB83
DIEA, DMSO
1
Pd/C
MeOH
2
Int.2, 2313
HATU, DIEA, DMF
2326

Synthesis of benzyl (1,29-bis((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-15-(1-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-10-oxo-3,6-dioxa-9,11-diazatetradecan-14-yl)-10,20-dioxo-3,6,24,27-tetraoxa-9,11,19,21-tetraazanonacosan-15-yl)carbamate; 3TFA (**1**)

To a mixture of (2R,3R,4R,5R,6R)-5-[[4-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]pyrimidin-2-yl]amino]-2-(methoxymethyl)-6-propyl-tetrahydropyran-3,4-diol;2,2,2-trifluoroacetic acid (**XB83**, 3.55 eq, 25.0 mg, 0.045 mmol) and DIPEA (10.6 eq, 0.023 mL, 0.13 mmol) in DMSO (0.2 mL) at 45 °C was added a solution of benzyl N-[4-(imidazole-1-carbonylamino)-1,1-bis[3-(imidazole-1-carbonylamino)propyl]butyl]carbamate (1.00 eq, 7.8 mg, 0.013 mmol) in DMSO (165 uL) in portions over 6 hours. The mixture was stirred at 45 °C overnight and purified by prep. HPLC (5 - 14 -35% MeCN/water with 0.1% TFA) to give **1** as a white solid (TFA salt, 20.4 mg, yield: 77%). LCMS m/z 1748.3 [M + H]

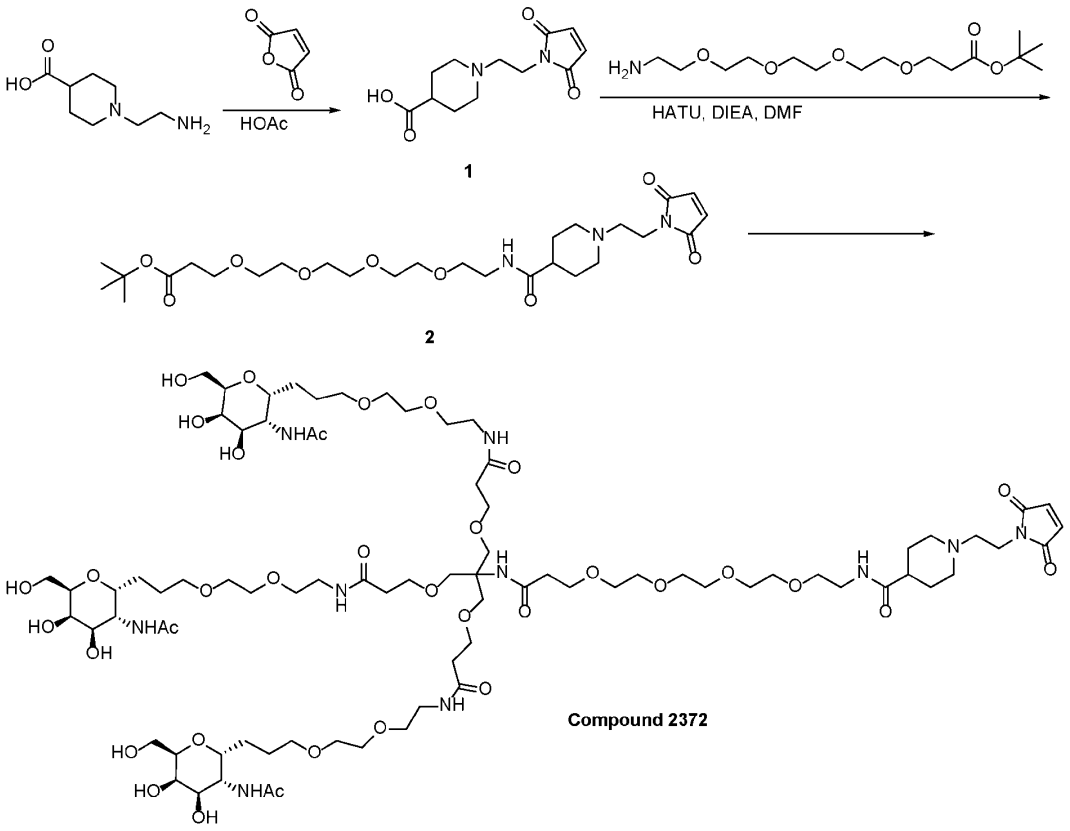
Synthesis of 1,1'-(4-amino-4-(1-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-10-oxo-3,6-dioxa-9,11-diazatetradecan-14-yl)heptane-1,7-diyl)bis(3-(2-(2-(2-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)ethoxy)ethoxy)ethyl)urea) (**2**)

To a mixture of benzyl (1,29-bis((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-15-(1-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-10-oxo-3,6-dioxa-9,11-diazatetradecan-14-yl)-10,20-dioxo-3,6,24,27-tetraoxa-9,11,19,21-tetraazanonacosan-15-yl)carbamate; 3TFA (**1**) (1.00 eq, 20.4 mg, 0.0098 mmol) in MeOH (3 mL) was added 10% Pd/C (14.5 mg). The mixture was stirred at room temperature under hydrogen for 1.5 h and filtered. The filtrate was concentrated and dissolved in MeOH (3 mL). 10% Pd/C (19 mg) was added. The mixture was stirred at room temperature under hydrogen for 1.5h, filtered, concentrated, and purified by prep HPLC (3 - 70% MeCN/20 mM NH4OH aqueous solution) to give **2** as a white solid (10 .1 mg, yield: 64%). LCMS m/z 1613.5 [M+H].

Synthesis of N-(1,29-bis((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-15-(1-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-10-oxo-3,6-dioxa-9,11-diazatetradecan-14-yl)-10,20-dioxo-3,6,24,27-tetraoxa-9,11,19,21-tetraazanonacosan-15-yl)-3-(2-(2-(2-((4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)sulfonamido)ethoxy)ethoxy)ethoxy)propenamide; 3TFA (**2326**)

To a mixture of 3-[2-[2-[2-[[4-(2,5-dioxopyrrol-1-yl)phenyl]sulfonylamino]ethoxy]ethoxy]ethoxy]propanoic acid (1.50 eq, 2.1 mg, 0.0046 mmol) in DMF (0.15 mL) at room temperature was added DIEA (4.00 eq, 0.0021 mL, 0.012 mmol) , HATU (1.50 eq, 1.7 mg, 0.0046 mmol), followed by addition of a solution of 1,1'-(4-amino-4-(1-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)-10-oxo-3,6-dioxa-9,11-diazatetradecan-14-yl)heptane-1,7-diyl)bis(3-(2-(2-(2-((2-(((2R,3R,4R,5R,6R)-4,5-dihydroxy-6-(methoxymethyl)-2-propyltetrahydro-2H-pyran-3-yl)amino)pyrimidin-4-yl)oxy)ethoxy)ethoxy)ethyl)urea) (**2**) (1.00 eq, 4.9 mg, 0.0030 mmol) in DMF (0.15 mL). The mixture was stirred at room temperature for 1h, purified by prep. HPLC (5 - 13 -40% MeCN/water with 0.1% TFA), and re-purified by prep. HPLC (3 - 19 - 50% MeCN/water with 0.1% TFA) to give **2326** as a white solid (TFA salt, 2.52 mg, yield:35%). LCMS: m/z 1027.2 $[M+2H]^{++}$.

Synthesis of Compound 2372



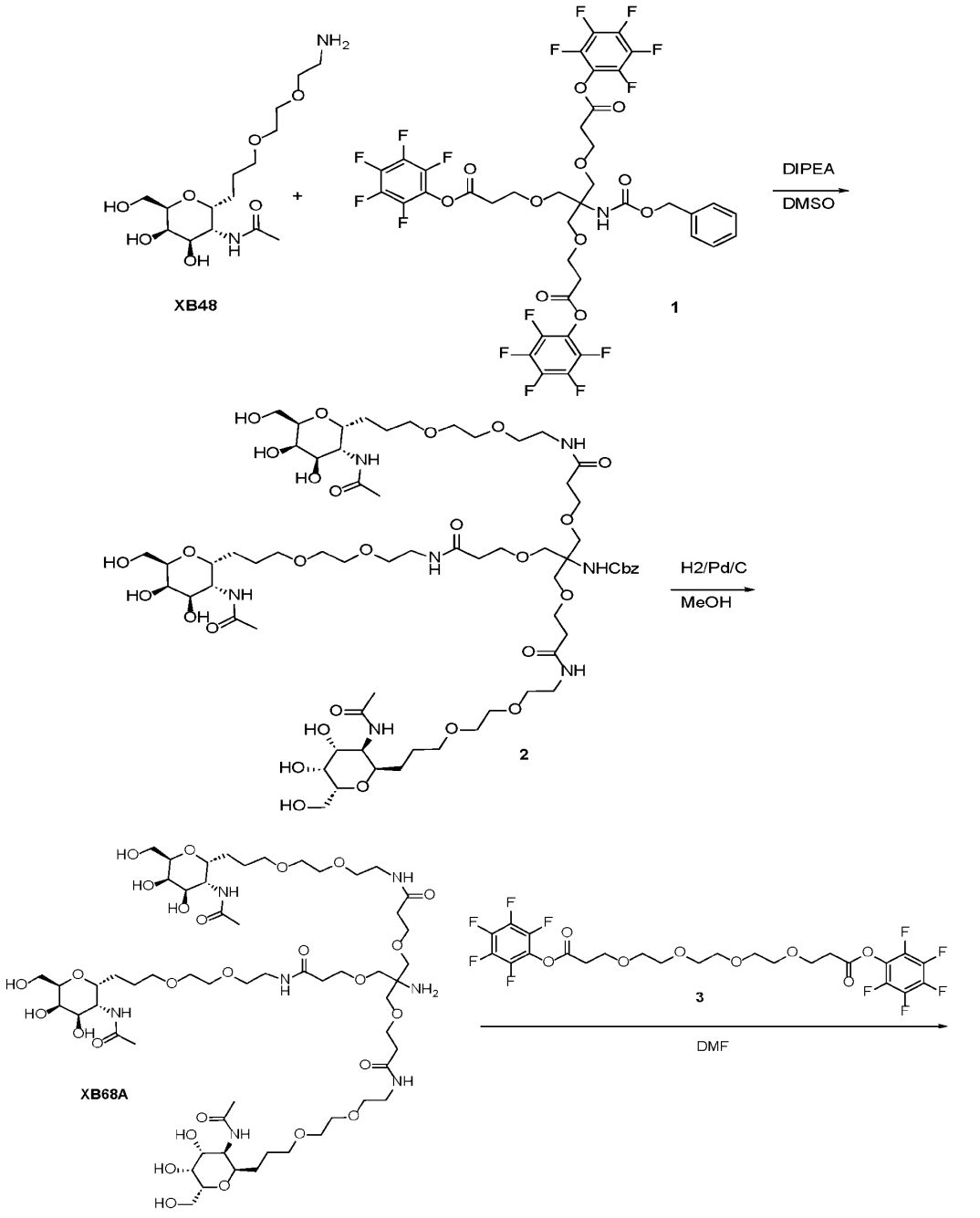
Synthesis of 1-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)piperidine-4-carboxylic acid (**1**): To a mixture of 1-(2-aminoethyl)piperidine-4-carboxylic acid (1.00 eq, 81.4 mg, 0.47 mmol) and maleic

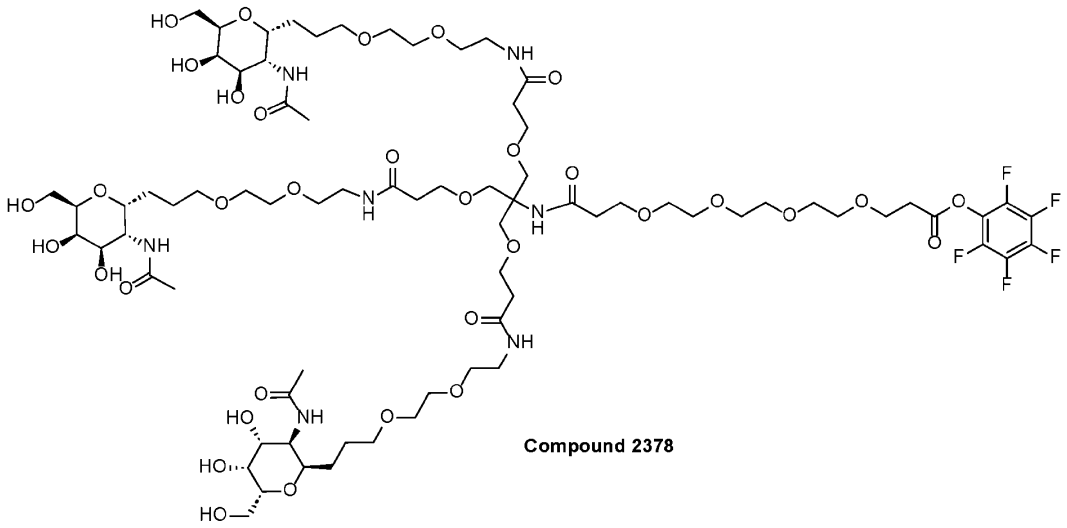
anhydride (1.00 eq, 46.3 mg, 0.47 mmol) was added HOAc (1.5 mL). The mixture was stirred at rt for 1h, concentrated, and co-evaporated with toluene (2x). To the residue was added toluene (1.5 mL). The mixture was stirred at 110 °C for 1hr and concentrated to give **1** as a pink solid which was used without purification. LCMS m/z 253.1 [M+H]

Synthesis of tert-butyl 1-(1-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)piperidin-4-yl)-1-oxo-5,8,11,14-tetraoxa-2-azaheptadecan-17-oate **(2):** To a mixture of crude 1-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)piperidine-4-carboxylic acid (0.47 mmol) in DMF (1.2 mL) at 0 °C were added HATU (1.30 eq, 234 mg, 0.62 mmol) and diisopropylethylamine (DIPEA) (2.50 eq, 0.21 mL, 1.18 mmol) , followed by addition of amino-PEG4-t-butyl ester (0.900 eq, 137 mg, 0.43 mmol) . The mixture was stirred at ambient temperature for 1hr and purified by prep. HPLC (5 - 40% MeCN/water with 0.1%TFA) to give **2** as clear syrup (51 mg, yield: 19% two steps). LCMS m/z 556.2 [M+H].

Synthesis of **Compound 2372:** Compound **2372** was synthesized by employing the procedures described for Compound **1251** using tert-butyl 1-(1-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)piperidin-4-yl)-1-oxo-5,8,11,14-tetraoxa-2-azaheptadecan-17-oate **(2)** in lieu of tert-butyl 12-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-12-oxododecanoate **(Compound 1251, 00A)** and using **XB48** in place of **XB47**. LCMS m/z 1816.5 [M+H].

Synthesis of Compound 2378





Compound 2378

Synthesis of benzyl (1,31-bis((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-16-(15-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-5-oxo-2,9,12-trioxa-6-azapentadecyl)-11,21-dioxo-4,7,14,18,25,28-hexaoxa-10,22-diazahentriacontan-16-yl)carbamate (**2**)

A mixture of N-((2R,3R,4R,5R,6R)-2-(3-(2-(2-aminoethoxy)ethoxy)propyl)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (**XB48**, 3.24 eq, 1.72 g, 4.91 mmol) and bis(perfluorophenyl) 3,3'-((2-(((benzyloxy)carbonyl)amino)-2-((3-oxo-3-(perfluorophenoxy)propoxy)methyl)propane-1,3-diyl)bis(oxy))dipropionate (1.00 eq, 1.47 g, 1.52 mmol) was treated with diisopropylethylamine (**1**, 3.00 eq, 862 mL, 4.95 mmol) then dissolved in DMSO (6.89 mL) with sonication. After 90 minutes the reaction was purified by reversed-phase HPLC (5% then 15-50% acetonitrile in water w/0.1% FA) to give **2**. Yield: 2.21 g, 91%. LCMS 1468.6 [M+H].

Synthesis of N-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethyl]-3-[3-[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]-2-[[3-[2-[2-[3-[(2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]propoxy]ethoxy]ethylamino]-3-oxo-propoxy]methyl]-2-amino-propoxy]propenamide (**XB68A**).

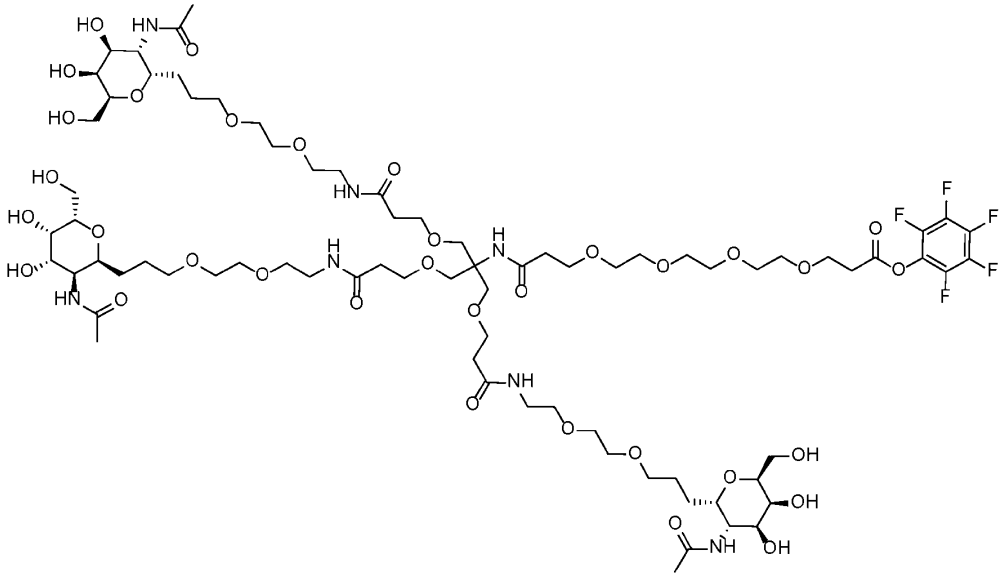
A solution of **2** (1.00 eq, 400 mg, 0.272 mmol) in methanol (20 mL) was purged with nitrogen then treated with 10% Pd/C (0.400 eq, 232 mg, 0.109 mmol) before being evacuated then back-filled with hydrogen via balloon. After 2h, the reaction was treated with 500 mg celite then filtered over a pad of

celite. The filter cake was rinsed with methanol and the filtrate was concentrated under reduced pressure. The residue was dissolved in water with minimal DMSO then purified by reversed-phase HPLC (5-50% acetonitrile in water w/ 0.2 mM NH_4OH) to give **XB68A**. Yield: 315 mg, 86.7%. LCMS 1335.6 [M+H].

Synthesis of perfluorophenyl 33-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-18,18-bis(15-((2R,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-5-oxo-2,9,12-trioxa-6-azapentadecyl)-16,23-dioxo-4,7,10,13,20,27,30-heptaoxa-17,24-diazatritriacontanoate (**Compound 2378**)

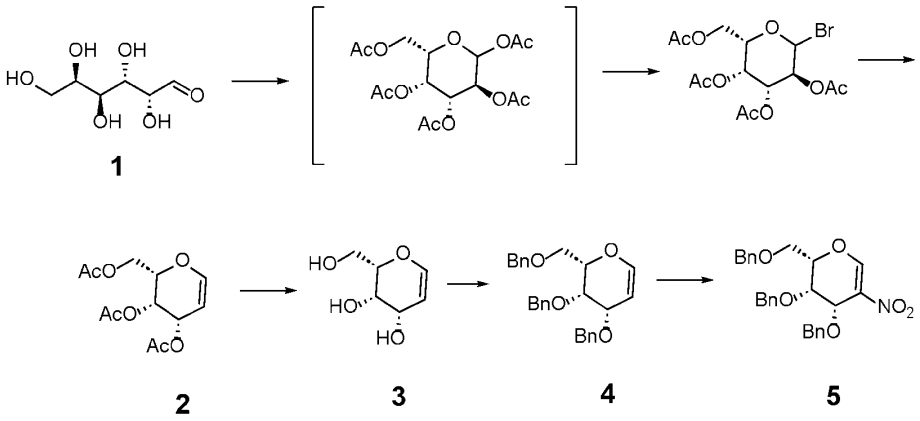
A solution of **XB68A** (1.00 eq, 1.10 g, 0.824 mmol) in DMF (13.7 mL) was added to a solution of bis(perfluorophenyl) 4,7,10,13-tetraoxahexadecanedioate (**3**, 3.00 eq, 1.55g, 2.47 mmol) in DMF (5.6 mL) drop-wise via syringe over 16 minutes. Next, DMF (2 mL) was added to the amine flask for rinsing then this solution was added to the reaction dropwise via syringe. The reaction was stirred at room temperature overnight. After 26h, the reaction was purified directly by reversed-phase HPLC (5-15-40% acetonitrile in water w/0.1% TFA) to give solids that were not easily manipulated. The residue was treated with acetonitrile to remove water via azeotropic distillation. The residue was then dissolved in MeOH (1 volume) then diluted with DCM (95 volumes) then concentrated under high vacuum for 18h to give **Compound 2378** as a powder. Yield: 1050 mg, 71%. LCMS 1777.58 [M+H].

Synthesis of Compound *ent*-2378



***ent*-2378** was prepared analogously to 2378 using (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran as starting material in place of (2R,3R,4R)-3,4-

dibenzyloxy-2-(benzyloxymethyl)-5-nitro-3,4-dihydro-2H-pyran. (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran was prepared by the procedure below.



Synthesis of (2S,3S,4S)-2-(acetoxymethyl)-3,4-dihydro-2H-pyran-3,4-diyl diacetate (2)

To a solution of acetic anhydride (65 ml, 688 mmol, 6.3 eq) was slowly added 70% perchloric acid (1.9 mL, 22 mmol, 0.2 eq.), while keeping solution at ambient temperature. While stirring, (2R,3S,4S,5R)-2,3,4,5,6-pentahydroxyhexanal (1) (19.6 g, 108 mmol, 1.0 eq.) was added portion-wise as a solid over approximately 30 min. The reaction stirred at ambient temperature for approximately 2 hours, then the reaction mixture was cooled to 0-5C, and 33% w/w hydrogen bromide in acetic acid (66.0 mL, 377 mmol, 3.5 eq.) was added dropwise over approximately 30 min. The reaction continued to stir while warming to ambient temperature. After 2 hours, the reaction solution was diluted with dichloromethane, transferred to a separatory funnel, and washed 2x with H2O, 2x with 5% w/w aqueous sodium bicarbonate, and 1x with brine solution. The dichloromethane layer was dried over sodium sulfate and solvent evaporated to afford crude (2S,3R,4R,5S)-2-(acetoxymethyl)-6-bromotetrahydro-2H-pyran-3,4,5-triyl triacetate intermediate as a dark brown color syrup (50.7 mg). The crude intermediate was dissolved in 50% v/v acetic acid/H2O (150 mL), stirred while cooling to approximately -20C, and zinc dust (35.8 g, 548 mmol, 5.0 eq.) was added as a solid portion-wise over approximately 30 minutes. The reaction stirred while warming to ambient temperature. After approximately 30 minutes, solids were removed by filtration over diatomaceous earth, followed by washing with dichloromethane. The filtrate was transferred to a separatory funnel, layers separated, and lower dichloromethane layer washed 2x with H2O, 1x with brine solution, dried over sodium sulfate, and solvent evaporated to crude syrup. The product was isolated by silica chromatography, eluting with 0-10% ethyl acetate/dichloromethane. Fractions containing product were combined, solvent evaporated, and dried under high-vacuum at

ambient temperature to afford (2S,3S,4S)-2-(acetoxymethyl)-3,4-dihydro-2H-pyran-3,4-diyl diacetate (2) as a clear syrup. Yield: 15.1 g (51%); LCMS m/z 295.03 [M+Na+].

Synthesis of (2S,3S,4S)-2-(hydroxymethyl)-3,4-dihydro-2H-pyran-3,4-diol (3)

A solution of (2S,3S,4S)-2-(acetoxymethyl)-3,4-dihydro-2H-pyran-3,4-diyl diacetate (2) (14.9 g, 54.7 mmol, 1.0 eq.), dissolved in methanol (150 mL), was cooled to 0-5C while stirring, followed by slow addition of 25% w/w sodium methoxide in methanol (3.8 mL, 16.6 mmol, 0.3 eq.). The reaction mixture then stirred while warming to ambient temperature. After approximately 15 min, the reaction mixture was evaporated onto silica and product isolated by silica chromatography, eluting with 70-100% ethyl acetate/dichloromethane, followed by product elution with 0-20% methanol/ethyl acetate. Fractions containing product were combined, solvent evaporated, and dried under high-vacuum at ambient temperature to afford (2S,3S,4S)-2-(hydroxymethyl)-3,4-dihydro-2H-pyran-3,4-diol (3) as a white solid. Yield: 6.9 g (86%); LCMS m/z 169.0 [M+Na+].

Synthesis of (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-3,4-dihydro-2H-pyran (4)

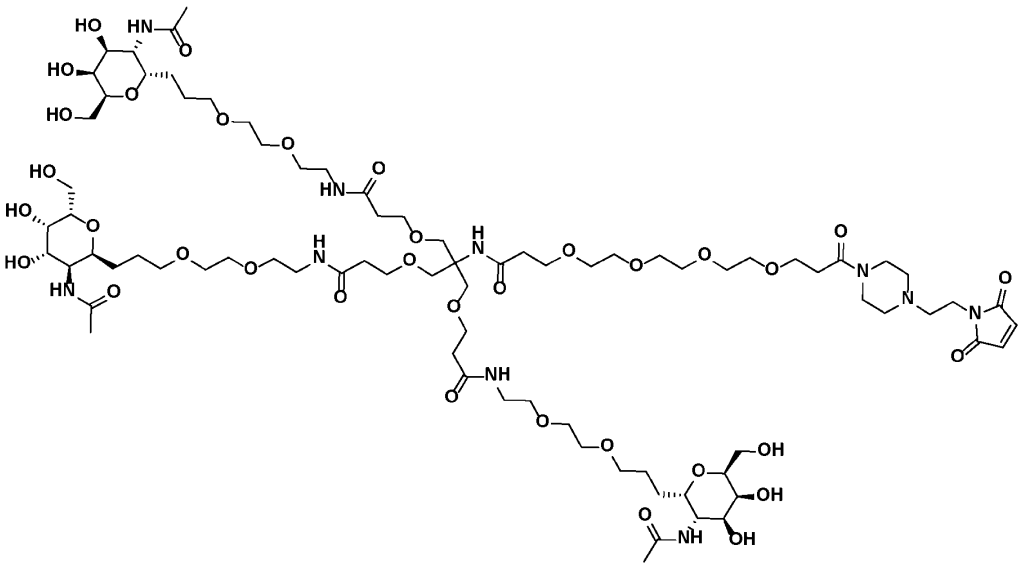
A solution of (2S,3S,4S)-2-(hydroxymethyl)-3,4-dihydro-2H-pyran-3,4-diol (3) (6.61 g 45.2 mmol, 1.0 eq.), dissolved in 60 mL of DMF, was stirred while cooling to 0-5C, followed by portion-wise addition of sodium hydride (60% dispersion in mineral oil) (7.28 g, 182 mmol, 4.0 eq). The slurry mixture continued to stir at 0-5C for approximately 40 min, then benzyl bromide (22.0 mL, 185 mmol, 4.0 eq.) was added dropwise over approximately 25 minutes. The reaction slurry continued to stir while warming to ambient temperature. After approximately 90 minutes, the reaction slurry was again cooled to 0-5C and quenched with dropwise addition of methanol (7 mL). The reaction quench continued to stir for approximately 15 minutes, then was diluted with ethyl acetate, transferred to separatory funnel, and washed 2x with cold H2O, 1x with brine solution, dried over sodium sulfate, and solvent evaporated to crude syrup. The product was isolated by silica chromatography, eluting with 0-50% ethyl acetate/hexanes. Fractions containing product were combined, solvent evaporated, and dried under high-vacuum at ambient temperature to afford (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-3,4-dihydro-2H-pyran (4) as a white solid. Yield: 15.8 g (84%); LCMS m/z 439.2 [M+Na+].

Synthesis of (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran (5)

A solution of (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-3,4-dihydro-2H-pyran (4) (9.70 g, 23.3 mmol, 1.0 eq.), in dichloromethane (200 mL), was added tetrabutylammonium nitrate (8.53 g, 28.0 mmol, 1.2 eq.). The solution stirred under nitrogen atmosphere while cooling to 0-5C, then trifluoroacetic anhydride (3.94 mL, 28.3 mmol, 1.2 eq.) was added dropwise over approximately 15 minutes. The reaction continued to stir while warming to ambient temperature. After approximately 2

hours, the reaction solution was again cooled to 0-5C and triethylamine (4.0 mL, 29 mmol, 1.2 eq) was added dropwise over approximately 10 minutes. To the stirring reaction mixture was added ice water (200 mL), solution transferred to separatory funnel, and product extracted into dichloromethane (500 mL). The dichloromethane layer was separated, washed 1x with ice water, 1x with brine solution, dried over sodium sulfate and concentrated to crude yellow colored oil. The product was isolated by silica chromatography, eluting with 0-20% ethyl acetate/hexanes. Fractions containing product were combined, solvent evaporated, and dried under high-vacuum at ambient temperature to afford (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran (5) as a clear slight yellow colored oil. Yield: 6.13 g (57%); 1H NMR (400 MHz, CDCl3) δ 7.44 – 7.27 (m, 15H), 4.98 – 4.82 (m, 3H), 4.82 – 4.44 (m, 6H), 4.04 – 3.91 (m, 3H).

Synthesis of Compound *ent*-1248



***ent*-1248** was prepared analogously to **1248** using (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran as starting material in place of (2R,3R,4R)-3,4-dibenzyloxy-2-(benzyloxymethyl)-5-nitro-3,4-dihydro-2H-pyran. (2S,3S,4S)-3,4-bis(benzyloxy)-2-((benzyloxy)methyl)-5-nitro-3,4-dihydro-2H-pyran was prepared by the procedure below.

Example 5: LYTACs and LYTAC Formats

LYTACs described herein may be of one of several formats. **Figs. 1A-B** show two possible formats. **Fig. 1A** shows an Fc fusion format in which TSHR protein 10 is linked to Fc, 20 via a polypeptide linker consisting of GGGGSGGGGSGGGGS (SEQ ID NO: 16). Internalizing receptor

ligand, 30, and linker, 40, are chemically conjugated (i.e. covalently bound) to the TSHR protein or the Fc fragment, or both. **Fig. 1B** shows an HSA fusion format in which TSHR protein 10 is linked to HSA 50 via a polypeptide linker consisting of GGGGSGGGGSGGGGS (SEQ ID NO: 16). Internalizing receptor ligand, 30, and linker, 40, are chemically conjugated (i.e. covalently bound) to the TSHR protein or the HSA protein, or both. Chemical conjugates may include attachment to either cysteines using thiol reactive linkers or lysines using amine reactive linkers. Other suitable formats of TSHR fusions with a carrier protein include single-chain Fc fusions, heterodimeric Fc fusions, and albumin binding domains. Regarding conjugation of the ligand-linker, conjugation may be at lysine residues as shown, or at cysteine residues. Details of conjugation are provided below.

Fc and HSA fusions as described in **Fig. 1A** and **Fig. 1B** were generated in various mammalian host cell expression secretion systems such as HEK-293 or CHO. For Fc fusions, the conditioned media was clarified via a 0.22um filter followed by purification on Protein A resin (Cytiva - mAb select sure) using an FPLC (Cytiva), in which following loading in accordance with manufacturers recommendations, the column was rinsed with an isotonic buffer such as phosphate buffered saline (PBS) (pH 7.5), followed by elution with 3 column volumes of 100mM acetic acid (pH 3.5), followed by neutralization of eluted pool to pH 5.5 with addition of 1M Tris pH 8.0. For HSA fusions, the conditioned media was clarified via a 0.22um filter followed by purification on Capture Select Human Albumin affinity matrix (Thermo Scientific) using an FPLC (Cytiva), in which following loading in accordance with manufacturers recommendations, the column was rinsed with an isotonic buffer such as Phosphate buffered saline (pH 7.5), followed by elution with 3 column volumes of 100mM glycine (pH 3.2), followed by neutralization of eluted pool to pH 8.0 with addition of 1M Tris pH 8.5. Following affinity capture of either Fc and HSA fusions, each protein was optionally further polished by FPLC using either size exclusion chromatography (Superdex 200 HiLoad 26/60, PBS pH 7.5) or via IEC (ion exchange chromatography) using SP-HP resin (Cytiva) at pH 5.0-5.5 with 20 column volumes of 1M NaCl gradient or using Q-HP resin (Cytiva) at pH 8.0-8.5 with 20 column volumes of 1M NaCl gradient. In all cases, the purified protein fractions were pooled via absorbance at 280nm wavelength.

Conjugation of Ligand-Linkers to Lys Residues

Ligand-linkers that include a pentafluorophenyl (PFP) ester were used to conjugate to lysine residues. The protein was buffer exchanged into 100 mM Borate, 50 mM NaCl, 1 mM EDTA, pH 8.3 (BBSE), after which the proper number of molar equivalents of PFP-based ligand-linker compound (e.g., freshly prepared as 20-30 mM stock solution in DMA) was added to achieve the desired Ligand-linker to Protein Ratio (LPR) and incubated for 3-18 hours at 25 °C or 30 °C (e.g., 7-8 molar equivalents for a LPR

of about 5 for TSHR-Fc and TSHR-HSA). The conjugates were purified using size exclusion chromatography (e.g. Superdex 200 Increase 10/300 GL column, Cytiva) and PBS pH 7.5.

Conjugation of Ligand-Linkers to Cys Residues

Ligand-linkers that include a maleimide reactive group were used to conjugate to cysteine residues. The protein was buffer exchanged into 100 mM borate, 50 mM NaCl, 1 mM EDTA, pH 8.3 (BBSE), after which 2-6 molar equivalents of thiol-reactive ligand-linker compound (e.g., freshly prepared as 20-30 mM stock solution in DMA) was added and incubated for up to 24 hours at 37 °C. The conjugates were purified using size exclusion chromatography (e.g. Superdex 200 Increase 10/300 GL column, Cytiva) and PBS pH 7.5.

Characterization of LYTACs

After conjugation of the ligand-linkers, LYTACs were characterized as follows:

a) Concentration: The concentration of the LYTACs was determined using UV absorbance measurements on a ThermoFisher Nanodrop instrument and the extinction coefficient calculated from the protein's primary amino acid sequence.

b) Endotoxin content: The LYTACs were diluted to 1 mg/mL with water and the endotoxin content was measured using Limulus amebocyte lysate cartridges (Charles River).

c) Aggregate analysis: The aggregation profile of the LYTACs was evaluated by size exclusion high-performance liquid chromatography (SEC-HPLC). The LYTACs were diluted to 1 mg/mL with PBS and 10 µL were loaded onto a Zenix SEC-300 column (Sepax) using isocratic elution at a constant flow rate of 0.5 mL/min and 2X PBS as the mobile phase. Chromatograms were integrated based on elution time to calculate the purity of monomeric LYTAC species. All LYTACs described herein were at least 95% monomeric.

d) Deglycosylation procedure: For identity confirmation and determination of Ligand-linker to Protein Ratio (LPR), the protein (unconjugated and conjugated) was first deglycosylated under denaturing conditions with rapid PNGase F (New England Biolabs). Deglycosylated samples were subsequently analyzed by liquid chromatography-mass spectrometry (LC-MS) and hydrophilic interaction liquid chromatography- high-performance liquid chromatography (HILIC-HPLC).

e) Determination of Ligand-linker to Protein Ratio (LPR) by LC-MS: The identity and LPR were determined via liquid chromatography-mass spectrometry (LC-MS). The protein (unconjugated and conjugated) was deglycosylated as described above and diluted 10-fold in 0.2% formic acid and analyzed on a Waters ACQUITY UPLC interfaced to Xevo G2-S QToF mass spectrometer. Deconvoluted masses

were obtained using Waters MassLynx 4.2 Software and LPR values were calculated using a weighted average of the peak intensities corresponding to each ligand-linked species using the following formula:

$$LPR = \frac{\sum_{i=1}^{n}(number\ of\ ligand - linkers)_i * (peak\ intensity)_i}{\sum_{i=1}^{n}(peak\ intensity)_i} \quad (Eq.\ 1)$$

f) Determination of LPR by HILIC-HPLC: The LPR was also determined using hydrophilic interaction liquid chromatography (HILIC) as an orthogonal method. The LYTAC was deglycosylated as described above and 5 µg were injected into a high-performance liquid chromatography (HPLC) instrument (Agilent) equipped with an amide-HILIC column (Waters). Solvent A consisted of 100% acetonitrile and 0.1% trifluoroacetic acid (TFA), and solvent B consisted of water and 0.1% TFA. A gradient elution from 30% B to 40% B was applied at 0.2 mL/min and monitored at 210nm and 280nm. LPR values were calculated using a weighted average of the peak heights corresponding to each ligand-linked species using the formula described herein (Eq.1).

Details of LYTACs and various test articles described herein are summarized in Table 13 and 14:

Table 13: LYTACs

LYTAC No.	Format	TSHR SEQ.	Fusion Protein Seq (Y-B)	Conjugation Sites	Ligand-Linker Precursor	LPR
21-L001	Fc fusion	SEQ ID NO: 4	SEQ ID NO:17	Lys	**1209**	4-6
21-L002	HSA fusion	SEQ ID NO: 5	SEQ ID NO:18	Cys	**1248**	1
21-L003	HSA fusion	SEQ ID NO:7	SEQ ID NO:19	Cys	**1248**	1
21-L004	HSA fusion	SEQ ID NO:6	SEQ ID NO:20	Lys	**2378**	4-6

Table 14: Test Articles (Experimental Controls)

Test Article No.	Format	TSHR SEQ.	Conjugation Sites	Ligand-Linker Precursor	LPR
21-TA1	Fc fusion	SEQ ID NO: 1	None/Mock unconjugated	-	-
21-TA2	Fc fusion	SEQ ID NO: 4	None/Mock unconjugated	-	-

Test Article No.	Format	TSHR SEQ.	Conjugation Sites	Ligand-Linker Precursor	LPR
21-TA3	HSA fusion	SEQ ID NO: 5	None/Mock unconjugated	-	-
21-C002	HSA fusion	SEQ ID NO: 5	Cys	***ent*-1248**	1
21-TA4	HSA fusion	SEQ ID NO:6	None/Mock unconjugated	-	-
21-C003	HSA fusion	SEQ ID NO: 6	Cys	***ent*-1248**	1
21-C004	HSA fusion	SEQ ID NO: 6	Lys	***ent*-2378**	4-6

Example 6: Expression and Stability of TSHR-Fc fusions

Three approaches were used to evaluate the expression and stability of TSHR-Fc fusion proteins.

To compare the expression of TSHR-Fc fusions, the conditioned media of transiently transfected Expi-293 (Thermo Fisher) cells were harvested and the total protein titer was measured using high performance liquid chromatography (HPLC). Titers of conditioned media were assessed via sequential injections of known volumes onto a Thermo Scientific MAbPac Protein A column 4x35mm using an Agilent 1200 series HPLC using a loading/wash mobile phase of 1X Phosphate buffered saline (pH 7.4) and were eluted using a step gradient of 100mM Acetic Acid pH 3.5. The AUC of the eluted protein was quantified relative to a standard curve of TSHR-Fc (21-L002) and reported as mg/L of expression titer.

To better assess stability of heterologously expressed Fc fusion proteins, HPLC was performed to assess the percent of mononeric protein vs. aggregated protein. For Fc fusions, the conditioned media are clarified via a 0.22um filter followed by purification on Protein A resin (Cytiva - mAb select sure) using an FPLC (Cytiva), in which following loading in accordance with manufacturers recommendations, the column is rinsed with an isotonic buffer such as Phosphate buffered saline (PBS) (pH 7.5), followed by elution with 3 column volumes of 100mM acetic acid (pH 3.5), followed by neutralization of eluted pool to pH 5.5 with addition of 1M Tris pH 8.0. The Protein A pool was quantified by A280 extinction coefficient and 10ug was injected onto a Sepax ZENIX-C SEC-300 column using an Agilent 1200 series HPLC using a mobile phase of 1X Phosphate buffered saline (pH 7.4) with isocratic flow rate of 0.5mL/min. The elution time was evaluated relative to a SEC Molecular Weight standard for elution time of peaks from Protein A pools (Biorad gel filtration standard). Integration of AUC from the eluted peaks were assessed for higher order molecular weight aggregates vs monomer.

Finally, since the patient-derived pathogenic autoantibody M22 binds to a conformational epitope on TSHR that requires it to be correctly folded, a dot blot was performed to compare the ability of the TSHR-Fc fusions to adopt a correctly folded form competent to bind M22. Two microliters of conditioned media from transiently transfected HEK293 cells was spotted onto a nitrocellulose membrane (LI-COR # 926-31090) and allowed to dry for 30 mins. The blot was blocked with TBS blocking buffer (LI-COR # 927-60001) for 45 mins at room temperature and incubated with 1 μg/ml biotinylated M22 for 16 hrs at 4 degrees Celsius. The blots were washed 3 times with PBST (137 mM NaCl, 2.7 KCl, 10 mM sodium phosphate, 1.8 mM potassium phosphate, 0.01% Tween-20, pH 7.4) and incubated with a 1:2000 dilution of IR Dye 800CW Streptavidin (LI-COR # 926-32230) in TBS blocking buffer. The blot was then washed 3 times with PBST, 1 time with PBS and imaged using a LI-COR Odyssey Imaging System. Quantification was performed using the Empiria Studio software (LI-COR).

Figs. 2A-C demonstrate improved stability as compared to wild-type TSHR (as evaluated by results of an expression assay shown in **Fig. 2A** and an aggregation assay shown in **Fig. 2B**). **Fig 2C** shows that the stabilized form of TSHR, 21-TA2, is dramatically improved for binding to biotinylated M22 compared to WT TSHR, 21-TA1. Since 21-TA2 showed only minor improvement in expression compared to 21-TA1 **(Fig. 2A)**, this result indicates that the improved binding is due to stabilization of the properly folded form of TSHR.

Example 7: Internalization of Autoantibodies by Fc-Fusion TSHR LYTACs

A cellular uptake assay was performed to investigate the ability of stabilized TSHR Fc fusion LYTACs to internalize and uptake Graves' patient-derived autoantibodies in human cells. Approximately 50,000 HepG2 cells were seeded onto 96 well plates and incubated at 37 °C for 48 hrs. LYTACs were mixed with equimolar concentrations of patient-derived autoantibodies labeled with Alexa647 dye and incubated for 30 mins at 22 °C. A log3 serial dilution was performed covering a final concentration range from 150-5.67 nM. Experimental groups were treated for 2 hrs with the indicated LYTACs. Following incubation, the cells were washed with phosphate buffered saline, detached using 0.25% trypsin solution and analyzed by flow cytometry in the APC channel. Cellular uptake was measured by calculating the mean fluorescence intensity of cells incubated with the indicated LYTACs.

FIGs. 3A-B show LYTAC and ASGPR receptor dependent uptake of two Graves' patient-derived autoantibodies: LYTAC 21-L001 stimulated uptake of Alexa647-labeled M22 (Sanders, *Lancet*, 2003) **(FIG. 3A)** and K1-70 (Evans, *Clinical Endocrinology*, 2010) autoantibodies **(FIG. 3B)**. Unconjugated TSHR-Fc showed minimal uptake of M22 and K1-70 and no uptake was observed in HepG2 ASGPR1/2 knockout cells with either LYTAC or unconjugated protein.

Example 8: Internalization of Autoantibodies

To confirm that signal from the cellular uptake assays described in Example 3 was the result of internalization of target antibodies into an acidified endosome compartment, an internalization assay was performed using M22 labeled with pH sensitive pHrodo green dye. Approximately 15,000 HepG2 cells were seeded onto a 96 well plate and incubated at 37 °C for 48 hrs. Cells were then treated with 100 nM of M22-pHrodo green and test articles as described below. The plate was placed in an Incucyte S3 machine and images were collected in the phase and green channels every 30 mins for 24 hrs. Internalization was measured by calculating the integrated green object intensity of intracellular punctae for each image.

FIG. 4 shows LYTAC and ASGPR receptor dependent internalization of M22-pHrodo into an acidified endosome compartment. LYTAC 21-L001 stimulated internalization of M22-pHrodo over the observed time course. Unconjugated TSHR-Fc showed minimal internalization of M22-pHrodo and no internalization was observed in HepG2 ASGPR1/2 knockout cells with either conjugated LYTAC or unconjugated protein.

Example 9: Removal of Furin Consensus Sequence

To test if adding the K250Q mutation to remove the furin consensus site impacted the ability of a LYTAC to internalize a Graves' patient-derived autoantibody, an uptake assay was performed comparing the uptake activity of LYTAC with or without the K250Q mutation. Approximately 50,000 HepG2 cells were seeded onto 96 well plates and incubated at 37 °C for 48 hrs. LYTACs were mixed with equimolar concentrations of patient-derived autoantibodies labeled with Alexa488 dye and incubated for 30 mins at 22 °C. A log3 seral dilution was performed covering a final concentration range from 300-3.7 nM. Experimental groups were treated for 2 hrs with the indicated LYTACs. Following incubation, the cells were washed with phosphate buffered saline, detached using 0.25% trypsin solution and analyzed by flow cytometry in the FITC channel. Uptake was measured by calculating the mean fluorescence intensity of cells incubated with the indicated LYTACs.

Fig. 5 shows that LYTACs with the K250Q mutation performed equivalently to LYTACs that did not contain this mutation. No difference was observed in M22 uptake between LYTACs with and without the K250Q mutation. No uptake was observed with either protein conjugated to an inactive enantiomer (21-C002, 21-C003) and minimal uptake was observed in ASGPR1/2 knockout HepG2 cells. These results show that removing the furin consensus sequence for improved manufacturability by adding the K250Q mutation had no impact on LYTAC activity *in vitro*.

Example 10: Cellular Uptake of K1-18 Antibodies

To confirm LYTACs were active for uptake against a patient-derived pathogenic autoantibody a cellular uptake assay was performed. Approximately 50,000 HepG2 cells were seeded onto 96 well plates and incubated at 37 °C for 48 hrs. TSHR-based LYTAC was mixed with an equimolar concentration of the patient-derived pathogenic autoantibody K1-18 (Evans, Clinical Endocrinology, 2010) labeled with Alexa647 dye and incubated for 30 mins at 22 °C. A log3 seral dilution was performed covering a final concentration range from 300-3.7 nM. Experimental groups were treated for 2 hrs with the indicated LYTACs. Following incubation, the cells were washed with phosphate buffered saline, detached using 0.25% trypsin solution and analyzed by flow cytometry in the APC channel. Uptake was measured by calculating the mean fluorescence intensity of cells incubated with the indicated LYTACs.

Fig. 6 shows that LYTAC mediated uptake of K1-18 and requires (1) a ligand capable of binding to ASGPR and (2) ASGPR itself. LYTAC 21-L003 effected uptake of K1-18 across the dose response. No uptake was observed for LYTAC conjugated to inactive enantiomer (21-C003) or in ASGPR1/2 knockout HepG2 cells.

Example 11: Binding to Thyroid Stimulating Hormone (TSH)

To determine if a LYTAC would bind to the thyroid stimulating hormone, a bio-layer interferometry (BLI) study on an unconjugated test article was performed. Measurements were made on the GatorPrime (Gator Bio) using anti-mouse IgG Fc probes (MFC; Gator Bio). The probes were equilibrated for 10 minutes in K Buffer (Gator Bio) and regenerated according to the manufacturer guidelines before use.

A dilution of mouse monoclonal anti-HSA antibody was prepared at 1mg/mL in K Buffer and loaded onto the MFC probes. After a baseline step, the probes were dipped into wells containing 150nM 21-TA4 until saturated for the first association step. Following another baseline step, probes were dipped into wells containing 5nM or 75nM TSH-His (R&D Systems) for the second association step. An absence of a shift during the second association step suggests that there is no binding between LYTACs and TSH-His. Full step details can be found in **Table 15**.

Table 15

Step	Reagent	Time (sec)
Baseline	K Buffer	120
Loading	mouse monoclonal anti-HSA Ab (1mg/mL)	90
Baseline	K Buffer	120
Association #1	21-TA4 (150nM)	150

Step	Reagent	Time (sec)
Baseline	K Buffer	120
Association #2	TSH-His (5nM or 75nM)	120

Fig. 7 shows that anti-HSA was loaded onto the anti-human Fc probe (loading) and that 21-TA4 was captured by immobilized anti-HSA in the first association step by the observed shifts at both stages. No additional shift was observed after adding TSH-His at either 5 nM or 75 nM. These results show that 21-TA4 does not bind TSH in vitro and will therefore is not expected to compete for TSH binding *in vivo*.

Example 12: Binding to Patient-Derived Antibodies

BLI experiments were conducted to measure the binding kinetics of 21-TA4 to patient-derived autoantibodies M22 and K1-18 BLI. Measurements were measurements on the GatorPrime (Gator Bio) using anti-human IgG Fc gen II probes (HFCII; Gator Bio). The probes were equilibrated for 10 minutes in K buffer (Gator Bio) and regenerated according to the manufacturer guidelines before use.

Dilutions of two pAAbs (K1-18 and M22) were prepared at 0.5 mg/mL in K buffer and loaded onto the HFCII probes. After a baseline step, probes were dipped into wells containing a 1:1 serial dilution of 21-TA4 with a top concentration of 50nM. Probes were dipped into wells containing K Buffer for the dissociation step. Binding kinetics were determined using the GatorOne K Analysis software. Full step details can be found in **Table 16**.

Table 16

Step	Reagent	Time (sec)
Baseline	K Buffer	120
Loading	pAAbs (K1-18 or M22)	120
Baseline	K Buffer	120
Association	21-TA4	180
Dissociation	K Buffer	180

Figs. 8A-B shows that 21-TA4 binds both K1-18 (**FIG. 8A**) and M22 (**FIG. 8B**) with different binding kinetics. Concentration-dependent shifts were observed for both antibodies resulting in binding curves that could be fit to a 1:1 binding model in the GatorOne K Analysis software. Observed kinetics resulted in estimated KD values of 4.3 nM and 18.1 nM for 21-TA4 binding to K1-18 and M22, respectively.

Example 13: Increasing LPR Increases Cellular Uptake

To evaluate if LYTACs with increased linker-protein ratio (LPR) due to non-specific lysine conjugation resulted in improved target uptake in vitro, a cellular uptake assay was performed. Approximately 50,000 HepG2 cells were seeded onto 96 well plates and incubated at 37 °C for 48 hrs. TSHR-based LYTAC was mixed with an equimolar concentration of the patient-derived pathogenic autoantibody K1-18 labeled with Alexa647 dye and incubated for 30 mins at 22 °C. A log3 seral dilution was performed covering a final concentration range from 300-0.14 nM. Experimental groups were treated for 2 hrs with the indicated LYTACs. Following incubation, the cells were washed with phosphate buffered saline, detached using 0.25% trypsin solution and analyzed by flow cytometry in the APC channel. Uptake was measured by calculating the mean fluorescence intensity of cells incubated with the indicated LYTACs.

Fig. 9 shows that a LYTAC with conjugation at lysines and an LPR of 4-6 (21-L004) resulted in higher magnitude of K1-18 uptake across a dose response compared to a LYTAC generated from the same protein but conjugated at site-specific cysteine with an LPR of 1 (21-L003). No uptake was observed with unconjugated TSHR-HSA fusion protein 21-TA4.

Example 14: Lysosomal Degradation of a Pathogenic Autoantibody

To determine if internalization of a pathogenic autoantibody by a LYTAC resulted in degradation in the lysosome, a degradation assay was performed using K1-18 antibody conjugated to a cathepsin cleavable quenched fluorescent probe. When the internalized K1-18 antibody reaches the lysosome following internalization, cathepsin proteases will cleave the probe and liberate dye from quencher resulting in increased fluorescence. To assess degradation, 15,000 HepG2 cells were seeded into each well of a 96 well plate and incubated for 48 hrs at 37 °C. LYTAC 21-L004 was mixed with cathepsin probe labeled K1-18 at an equimolar concentration and a log2 serial dilution was performed cover a final concentration range from 150-2.34 nM in complete growth media (EMEM with 10% fetal bovine serum) and added to the cells. Degradation was monitored using the Incucyte S3 system with images acquired in the phase and red channel every 30 mins for 7 days. Degradation was quantified by measuring total red object integrated intensity (red calibrated unit – RCU per square μm per image) for fluorescent intracellular punctae.

Figs. 10A shows LYTAC-mediated degradation of K1-18 across over a 1 wk time course as indicated by increased total red object integrated intensity across the LYTAC:K1-18 dose response. As shown in **Fig. 10B**, by calculating the area under the kinetic curve at each dose level, it was observed that LYTAC 21-L004 was active across the full LYTAC dose range from 2.34 to 150 nM with activity

approaching saturation above 100 nM. These results indicate that LYTAC treatment resulted in degradation of the pathogenic antibody in a lysosome following cellular internalization.

Example 15: Degradation of Pathogenic Autoantibodies in Hepatocytes

To test the ability of LYTACs to mediate internalization and degradation of pathogenic autoantibodies in primary human hepatocytes, a degradation assay was performed using K1-18 antibody conjugated to a cathepsin-cleavable quenched fluorescent probe. When the internalized K1-18 antibody reaches the lysosome following internalization, cathepsin proteases will cleave the probe and liberate dye from quencher resulting in increased fluorescence. To assess degradation, 350,000 primary hepatocyte cells (Xenotech) were seeded into each well of a 24 well plate and incubated for 16 hrs at 37 °C. LYTAC 21-L004 was mixed with cathepsin probe labeled K1-18 for a final concentration of 100 nM each in primary hepatocyte growth media (OptiCulture, Xenotech) and added to the cells. Degradation was monitored using the Incucyte S3 system with images acquired in the phase and red channel every 30 mins for 3 days. Degradation was quantified by measuring total red object integrated intensity (red calibrated unit – RCU per square μm per image) for fluorescent intracellular punctae.

Fig. 11 shows that treatment of primary hepatocytes with LYTACs results in degradation of K1-18 autoantibodies as measured by increased fluorescence intensity due to dequenching of the cathepsin-cleavable probe conjugated antibody. Treatment with LYTAC 21-L004 resulted in increased K1-18 degradation compared to treatment with TSHR-HSA conjugated to a non-binding stereoisomer of the ASGPR ligand that does not bind ASGPR (21-C004) or probe labeled K1-18 alone.

Example 16: Depletion of anti-TSHR Autoantibodies from Patient Serum

An anti-TSHR antibody depletion assay was performed to test if TSHR LYTACs degrade patient-derived antibodies. LYTAC 21-L004 was immobilized on MyOne Tosylactivated Dynabeads (Thermo Fisher Scientific) according to the manufacturer's instructions Patient serum samples were thawed at room temperature and diluted by mixing 120 uL of serum with 240 uL of phosphate buffered saline, pH 7.4. 150 uL (0.3 mg/ml) of coated beads were added to the diluted serum samples and incubated at 4C for 16 hrs with gentle rotation. Beads were separated by a magnet and the supernatant was recovered as the post-depletion serum samples. Serum TSAb titers pre- and post-depletion were measured using a commercially available ELISA Kit (Kronus Inc.) according to the manufacturer's instructions.

Fig. 12 shows that TSAb levels for all patient serum samples tested (N=15) were depleted to below the limit of detection. These results indicate that the LYTAC 21-L004 can bind the full spectrum of TSAbs from the tested serum samples.

Example 17: In Vivo Clearance of Pathogenic Antibodies from Mouse Serum

A single-dose pharmacodynamics study was conducted to assess the ability of LYTAC 21-L004 to degrade three different anti-TSHR antibodies from circulation in mice. The study was conducted in an AALAC accredited facility under an IACUC approved protocol. Sixteen hours after the IV administration of the biotin-labeled antibodies, a serum sample was collected prior to dosing to measure pre-dose antibodies titers. A single bolus subcutaneous injection of LYTAC 21-L004 (1, 3, 10 or 30 mg/kg) or unconjugated protein (21-TA4) (30 mg/kg) was administered. Serum samples were collected at 4 hours post-dose and biotin-labeled anti-TSHR antibody levels were measured by ELISA. 96 well Maxisorp (Nunc) plates were coated with 2 µg/ml goat anti-human Fc antibody (Invitrogen #31125) in phosphate buffered saline (PBS – 137 mM NaCl, 2.7 KCl, 10 mM sodium phosphate, 1.8 mM potassium phosphate, pH 7.4) overnight at 4 degrees Celsius. The plate was washed 3 times with PBS containing 0.01% Tween-20 (Sigma Aldrich) (PBST) and incubated for 1 hr with PBST containing 1% bovine serum albumin (Sigma Aldrich) (BSA) to block non-specific protein binding. The blocking solution was aspirated, 50 µl diluted serum samples (log2 dilutions ranging from 1:20 to 2560 diluted in PBST with 1% BSA, 1:20 naïve mouse serum and 1 ug/ml THSR-HSA) were added to the wells and incubated for 1 hr at room temperature. The wells were washed 3 times with PBST and diluted streptavidin-HRP (Thermo Scientific #21130) (1:2000 in in PBST with 1% BSA) was added to each well and incubated 1 hr at room temperature. The wells were washed 3 times with PBST and the plates were developed with TMB substrate solution (Thermo Scientific #N301) for 5 mins. The reaction was stopped by adding stop solution for TMB substrates (Thermo Scientific #N600) and absorbance was measured at 450 nm in an Envision (Perkin Elmer) plate reader. Titers were determined using the empirically determined appropriate serum sample dilution and comparing to a standard curve generated using serially diluted biotinylated anti-TSHR antibody. The limit of detection was determined to be 31.25 ng/ml biotin-labeled anti-TSHR antibody.

Figs. 13A-F show dose-dependent clearance of all anti-TSHR antibodies tested from circulation at 4 hours following administration of LYTAC 21-L004. A 3 mg/kg dose resulted in statistically significant clearance (One-way ANOVA; $p<0.0001$) and a 10 mg/kg dose resulted in clearance to the limit of detection for all antibodies tested. The observed clearance depended on the ASGPR ligand since treatment with unconjugated protein (21-TA4) resulted in elevated (K1-18 and M22) or unchanged (IRI-SAB2) circulating antibody levels.

To confirm that the increase in anti-TSHR antibody titer observed 4 hrs following LYTAC treatment in single dose pharmacodynamics studies (**Fig. 13**) was due to redistribution from peripheral circulation/tissue and not due to an inability to fully clear anti-TSHR antibodies, an experiment was

performed in which the LYTAC was dose only 30 mins after the anti-THSR antibody to minimize antibody distribution within the animals. The study was conducted in an AALAC accredited facility under an IACUC approved protocol. A single bolus intravenous injection of Biotin-labeled anti-TSHR antibody (IRI-SAb2) at 0.1 mg/kg was administered into female BALB/c mice. Thirty minutes after the IV administration of IRI-SAb2, a serum sample was collected prior to dosing, and a single bolus intravenous injection of 21-L004 (10 mg/kg) was administered. Serum samples were collected at 0.083, 0.5, 2, 6, 24, 48, 72, and 96 hours post-dose and biotin-labeled anti-TSHR antibody levels were measured by ELISA.

Fig. 14 shows that a single 10 mg/kg dose of 21-L004 resulted in complete clearance of IRI-SAb2 from circulation to the limit of detection by 30 mins post-dosing. This experiment shows that 21-L004 is capable of clearing all circulating IRI-SAb2 antibodies available in circulation at the time of dosing.

Example 18: Clearance of a Polyclonal Mixture of Antibodies from Serum

To determine if 21-L004 can clear a polyclonal mixture of antibodies from serum an active immunization adoptive transfer model was developed. Balb/c mice were immunized via hock injection with 20ug JMG-HSA-His emulsified in Incomplete Freund's Adjuvant (Sigma Aldrich). The mice were boosted 14 days later with a second hock injection of 20ug Seq ID 2 emulsified in Incomplete Freund's Adjuvant. 28 days after the second immunization, the mice were euthanized and spleens harvested for lymphocyte isolation. Spleens were processed by mashing through a 70um filter followed by incubation with ACK buffer to lyse red blood cells. Lymphocytes were counted and resuspended in PBS at a concentration of 200 x 10^6/ml. Resuspended donor lymphocytes (20 x 10^6 per mouse) were then injected via tail vein into recipient NSG mice. Blood was drawn from recipient mice weekly to confirm engraftment and measure concentration of anti-TSHR antibodies. Recipient mice were normalized into treatment groups based on anti-TSHR titers and dosed subcutaneously with test articles (n=10/group) two weeks after cell transfer. Blood was drawn and serum isolated at indicated timepoints, with anti-TSHR IgG and LYTAC:Ab complex measured via ELISA.

Fig. 15 shows that 21-L004 cleared a mixture of anti-TSHR antibodies elicited from immunization with Seq ID 2. Shown on the left axis (closed circles) a single dose of 21-L004 cleared 70.5% of anti-TSHR from serum 4 hours after LYTAC dose. Since transferred B-cells are still generating anti-TSHR antibodies, the titers then increased over the course of 72 hrs reaching an average of 67% of the starting concentration. Shown on the right axis (open circles) a transient increase formation of complexes that include both LYTAC and anti-TSHR antibodies was observed 4 hours after dosing that was resolved to baseline signal by 24 hrs. These results demonstrate that L00493 treatment results in

clearance of a polyclonal mixture of anti-TSHR antibodies and that formation of circulating LYTAC:Ab complexes were transient consistent with a rapid clearance LYTAC mechanism of action.

Example 19: Serum Clearance of K1-18 Antibodies in hFcRn Mice

To test the in vivo behavior of 21-L004 in an organism with human FcRn, hFcRn Tg32 mice (The Jackson Laboratory) were used to evaluate K1-18 clearance following LCA-0321 treatment. Biotin-labeled K1-18 was IV administered at 0.1 mg/kg to hFcRn Tg32 mice. After 16 hours, a blood sample was collected for measurement of K1-18 levels, and mice were treated with LCA-0321 administered SC at 3 and 10 mg/kg or vehicle. Blood samples were collected at 4, 24, 48 and 72 hours post-treatment and biotin-labeled K1-18 concentrations were measured by ELISA. The results of this study are shown in **FIG. 16**. As shown in **FIG. 16**, biotin-K1-18 were cleared from serum 4 hours after the LCA-0321 treatment, at 3 or 10 mg/kg dose.

Example 20: Mapping of Lysine Conjugation Sites on TSHR-HSA protein construct

LC-MS Analysis of a Glu-C Digest of the TSHR-HSA-2378 conjugate was performed to identify conjugation sites and their prevalence.

Table 17. TSHR-HSA-2378 Lysine Conjugation Site Mapping

Peptide	Domain	Count	% of total
LVKHKPKATKE (SEQ ID NO:21)	HSA	1504708	27.47
LLFFAKRYKAAFTE (SEQ ID NO:22)	HSA	725319	13.24
IQRIPSLPPSTQTLKLIE (SEQ ID NO:23)	TSHR	638753	11.66
VSRNLGKVGSKC[+57.021]C[+57.021]KHPE (SEQ ID NO:24)	HSA	579677	10.58
RQIKKQTALVE (SEQ ID NO:25)	HSA	422626	7.72
TFLKKYLYE (SEQ ID NO:26)	HSA	378214	6.90
GKASSAKQRLKC[+57.021]ASLQKFGE (SEQ ID NO:27)	HSA	354094	6.46
NC[+57.021]DKSLHTLFGDKLC[+57.021]TVATLRE (SEQ ID NO:28)	HSA	326844	5.97
KC[+57.021]C[+57.021]KADDKE (SEQ ID NO:29)	HSA	98901	1.81
AVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQ.TSVTALPSKGLE (SEQ ID NO:30)	TSHR	81134	1.48
GKKLVAASQAALGL (SEQ ID NO:31)	HSA	73045	1.33
YSWLLLRLAKTYE (SEQ ID NO:32)	HSA	55118	1.01
NFKALVLIAFAQYLQQC[+57.021]PFEDHVKLVNE (SEQ ID NO:33)	HSA	44232	0.81

Peptide	Domain	Count	% of total
FKPLVEEPQNLIKQNC[+57.021]E (SEQ ID NO:34)	HSA	42414	0.77
QLKAVMDDFAAFVE (SEQ ID NO:35)	HSA	41822	0.76
KC[+57.021]C[+57.021]AAADPHE (SEQ ID NO:36)	HSA	34066	0.62
VAHRFKDLGEE (SEQ ID NO:37)	HSA	32011	0.58
C[+57.021]C[+57.021]QAADKAAC[+57.021]LLPKLDE (SEQ ID NO:38)	HSA	23369	0.43
LPLLKFLGIFNTGLKMFPPLTKVYSTE (SEQ ID NO:39)	TSHR	16548	0.30
AKDVFLGMFLYE (SEQ ID NO:40)	HSA	4724	0.09
	Total Lys Conjugation:	5477619	100
% ON HSA			86.56
% ON TSHR			13.44

The following amino acid sequences are referred to herein.

Table 18: LYTAC Sequences (Y-B fusion protein)

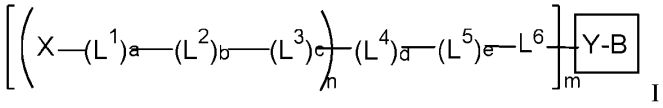
Amino acid sequence	SEQ ID NO:
MGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETHLRTIPSHAFSNLPNISRI YVSIDVTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPDALKELPLLKFLGIFNTGLKMF PDLTKVYSTDIFFILEITDNPYMTSIPVNAFQGLCNETLTLKLYNNGFTSVQGYAFNGTK LDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTALPSKGLEHLKELIARNTWTL (SEQ ID No:2)	17
GGGGSGGGGSGGGGS (SEQ ID NO:16)	
DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:13)	

Amino acid sequence	SEQ ID NO:
MGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETCLRTIPSHAFSNLPNISRI YVSIDVTLQQLESHSFYNLSKVTHIEIRNTPNLTYIDPDALKELPLLKFLGIFNTGLKMF PPLTKVYSTEIFFILEITDNPYMTSIPRNAFQGLCNETLTLKLYNNGFTSVQGYAFNGTK LDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTALPSKGLEHLKELRARNTWTL (SEQ ID No:5)	18
GGGGSGGGGSGGGGS (SEQ ID NO:16)	
DKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:13)	
GCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETHLRTIPSHAFSNLPNISRIY VSIDVTLQQLESHSFYNLSKVTHIEIRNTPNLTYIDPDALKELPLLKFLGIFNTGLKMFP PLTKVYSTEIFFILEITDNPYMTSIPRNAFQGLSNETLTLKLYNNGFTSVQGYAFNGTKL DAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTALPSKGLEHLKELRARNTWTL (SEQ ID NO:7)	19
GGGGSGGGGSGGGGS (SEQ ID NO:16)	
DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAE NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTK VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPA DLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKC CAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVST PTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTES LVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKAT KEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL (SEQ ID NO:15)	
GCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQTLKLIETHLRTIPSHAFSNLPNISRIY VSIDVTLQQLESHSFYNLSKVTHIEIRNTPNLTYIDPDALKELPLLKFLGIFNTGLKMFP PLTKVYSTEIFFILEITDNPYMTSIPRNAFQGLSNETLTLKLYNNGFTSVQGYAFNGTKL DAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTALPSKGLEHLQELRARNTWTL (SEQ ID NO:6)	20

Amino acid sequence	SEQ ID NO:
GGGGSGGGGSGGGGS (SEQ ID NO:16)	
DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAE NCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEV DVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLP KLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTK VHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPA DLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKC CAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVST PTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTES LVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKAT KEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL (SEQ ID NO:15)	

CLAUSES

Embodiment 1. A conjugate of Formula I:



I

or a prodrug thereof, or a salt thereof, wherein:

n is 1 to 10;

m is 1 to 10;

X is a moiety that binds to asialoglycoprotein receptor (ASGPR);

Y is a carrier polypeptide connected to B;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody;

each L^1 to L^6 is independently a linking moiety which together provide a linear or branched linker between each X and the Y-B complex; and

a, b, c, d, and e are each independently 1, 2, 3, 4, or 5.

Embodiment 2. The conjugate of embodiment 1, wherein B comprises an extracellular domain of TSHR or a fragment thereof.

Embodiment 3. The conjugate of embodiment 1 or 2, wherein B comprises a polypeptide having at least 80% (e.g., at least 85%, or at least 90%) sequence identity with a sequence of SEQ ID NOs: 1-7.

Embodiment 4. The conjugate of any one of embodiments 1-3, wherein B comprises a polypeptide having at least 95% sequence identity with a sequence of SEQ ID NOs: 1-7.

Embodiment 5. The conjugate of any one of embodiments 1-4, wherein B comprises a polypeptide having at least 98% sequence identity with a sequence of SEQ ID NOs: 1-7.

Embodiment 6. The conjugate of any one of embodiments 1-5, wherein B comprises a polypeptide of one of SEQ ID NOs: 1-7.

Embodiment 7. The conjugate of any one of embodiments 1-6, wherein B consists essentially of a polypeptide of one of SEQ ID NOs: 1-7.

Embodiment 8. The conjugate of embodiment 1, wherein B has one or more amino acid substitutions selected from R112P, D143P, D151E, V169R, C176S, K250Q, and I253R as compared to a sequence of SEQ ID NO:
1-7.

Embodiment 9. The conjugate of embodiment 1, wherein B has one or more amino acid substitutions selected from H63C, R112P, D143P, D151E, V169R, I253R as compared to a sequence of SEQ ID NO: 1-7.

Embodiment 10. The conjugate of any one of embodiments 1 to 9, wherein Y is selected from albumin, albumin binding domain, multimerization domain, Fc domain, Fc (monomer), Fc (dimer), fragments thereof (e.g., synthetic peptides), and variants thereof.

Embodiment 11. The conjugate of any one of embodiments 1 to 10, wherein Y comprises an albumin, a fragment thereof, or a variant thereof.

Embodiment 12. The conjugate of embodiment 11, wherein Y comprises human serum albumin (HSA), an HSA domain, bovine serum albumin (BSA), a fragment thereof, or a variant thereof.

Embodiment 13. The conjugate of embodiment 12, wherein Y is an HSA variant engineered for increased stability, conjugation efficiency (Cys or Lys), and/or FcRn binding.

Embodiment 14. The conjugate of embodiment 12, wherein Y comprises a polypeptide having 1 to 10 amino acid substitutions, deletions or additions (for example, 1, 2, 3, 4 or 5 amino acid substitutions, deletions or additions) as compared to a sequence of SEQ ID NOs: 8-12, 14 and 15.

Embodiment 15. The conjugate of embodiment 14, wherein the 1 to 10 amino acid substitutions, deletions or additions are selected from C34A, V54C, K93C, H128C, K262C, and E294C.

Embodiment 16. The conjugate of embodiment 12, wherein Y comprises a site-specific mutation of a naturally occurring amino acid residue.

Embodiment 17. The conjugate of embodiment 12, wherein Y comprises a polypeptide having at least 80% (e.g., at least 90%, at least 95%, at least 98%) sequence identity with a sequence of SEQ ID NOs: 8-12, 14 and 15.

Embodiment 18. The conjugate of embodiment 12, wherein Y comprises a polypeptide of one of SEQ ID NOs: 8-12, 14 and 15.

Embodiment 19. The conjugate of any one of embodiments 1 to 10, wherein Y comprises a Fc domain, a fragment thereof, or a variant thereof.

Embodiment 20. The conjugate of embodiment 19, wherein Y comprises Fc (monomer).

Embodiment 21. The conjugate of embodiment 19, wherein Y comprises Fc (dimer).

Embodiment 22. The conjugate of any one of embodiments 19 to 21, wherein Y comprises a Fc domain engineered for increased stability, conjugation efficiency (Cys or Lys), and/or FcRn binding.

Embodiment 23. The conjugate of embodiment 19, wherein Y comprises a polypeptide having 1 to 10 amino acid substitutions, deletions or additions (for example, 1, 2, 3, 4 or 5 amino acid substitutions, deletions or additions) as compared to a sequence of SEQ ID NO: 13.

Embodiment 24. The conjugate of embodiment 23, wherein the 1 to 10 amino acid substitutions, deletions or additions are selected from T366W, T366S, X368A, A378C, X407V, and L443C.

Embodiment 25. The conjugate of embodiment 19, wherein Y comprises a polypeptide having at least 80% (e.g., at least 90%, at least 95%, at least 98%) sequence identity with a sequence of SEQ ID NO: 13.

Embodiment 26. The conjugate of embodiment 19, wherein Y comprises a polypeptide of SEQ ID NO: 13.

Embodiment 27. The conjugate of any one of embodiments 1 to 26, wherein Y-B is a chimeric fusion protein.

Embodiment 28. The conjugate of embodiment 27, wherein Y-B is selected from an Fc-TSHR fusion, and an HSA-TSHR fusion.

Embodiment 29. The conjugate of embodiment 27, wherein Y is covalently linked to B via a linker.

Embodiment 30. The conjugate of embodiment 29, wherein the linker comprises a non-peptidic linking moiety.

Embodiment 31. The conjugate of embodiment 27, wherein Y is fused directly to B.

Embodiment 32. The conjugate of embodiment 31, wherein Y is fused indirectly to B via a spacer domain.

Embodiment 33. The conjugate of embodiment 31, wherein the N-terminal of Y is fused to the C-terminal of B.

Embodiment 34. The conjugate of embodiment 31, wherein the C-terminal of Y is fused to the N-terminal of B.

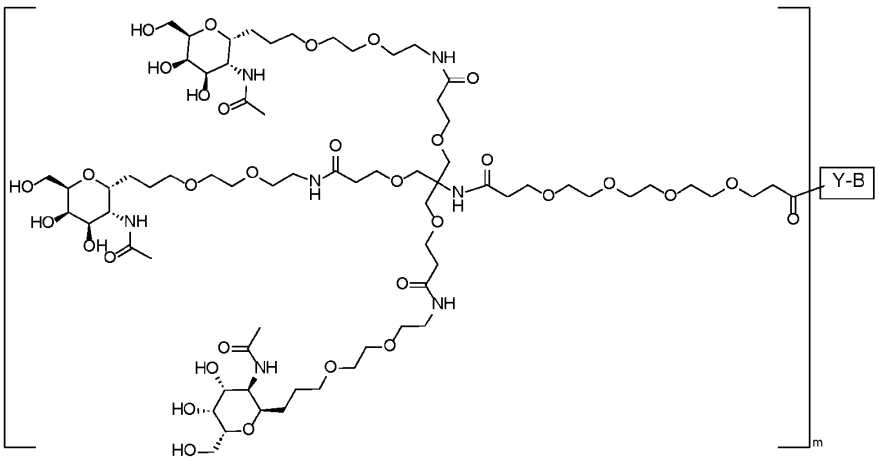
Embodiment 35. The conjugate of any one of embodiments 1 to 26, wherein Y is covalently linked to B via a non-peptidic linking moiety (e.g., a bifunctional linker).

Embodiment 36. The conjugate of any one of embodiments 1-35, wherein n is from about 2 to about 10.

Embodiment 37. The conjugate of any one of embodiments 1-36, wherein L is conjugated to one or more lysine residues of Y or B.

Embodiment 38. The conjugate of any one of embodiments 1-36, wherein L is conjugated to one or more cysteine residues of Y.

Embodiment 39. A conjugate of Formula:



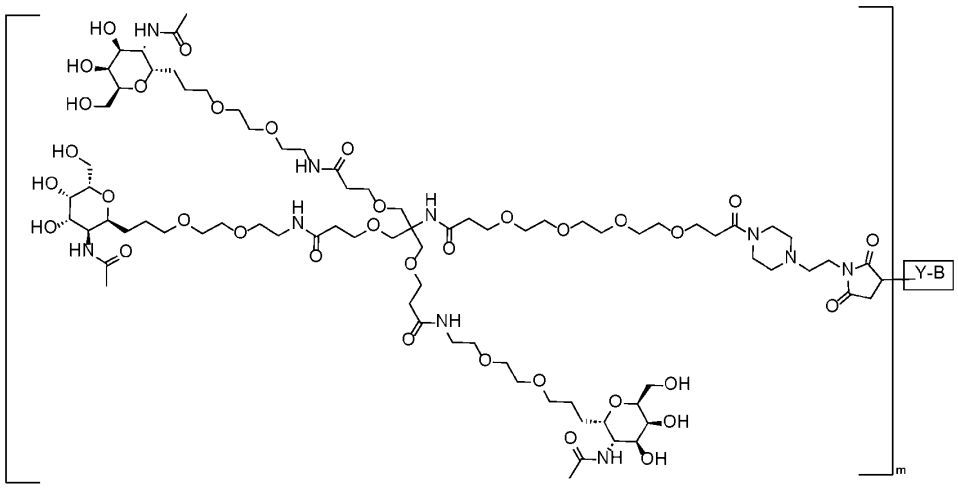
wherein:

m is 1 to 10;

Y is a carrier polypeptide connected to B; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody.

Embodiment 40. A conjugate of Formula:



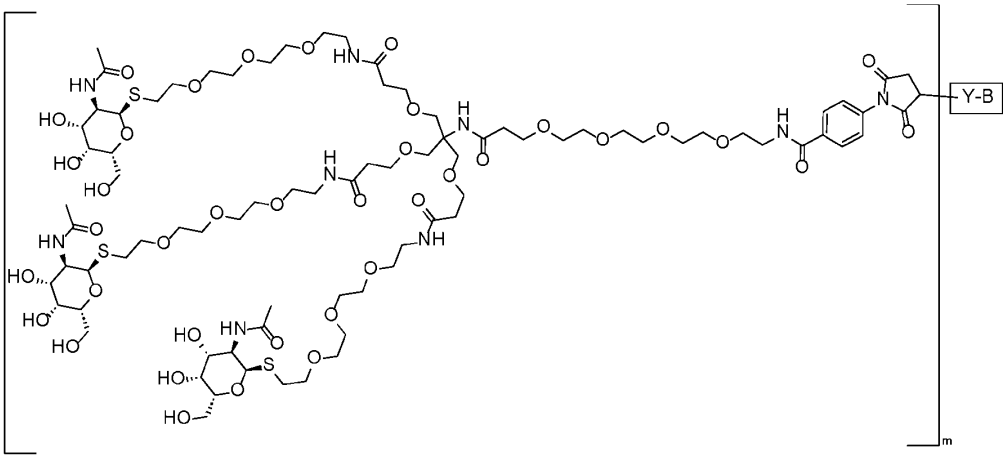
wherein:

m is 1 to 10;

Y is a carrier polypeptide connected to B; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody.

Embodiment 41. A conjugate of Formula:



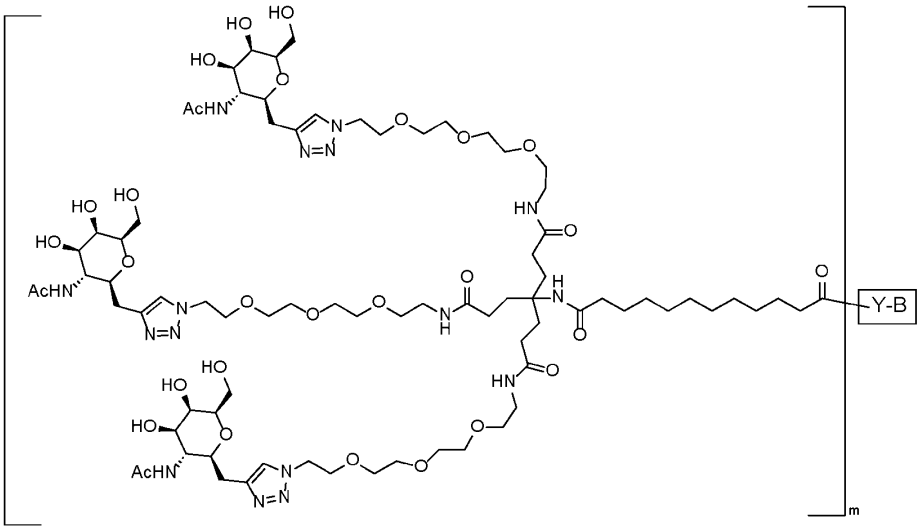
wherein:

m is 1 to 10;

Y is a carrier polypeptide connected to B; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody.

Embodiment 42. A conjugate of Formula:



wherein:

m is 1 to 10;

Y is a carrier polypeptide connected to B; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody.

Embodiment 43. The conjugate of any preceding embodiment, wherein Y is connected to B via a linking sequence.

Embodiment 44. The conjugate of embodiment 43, wherein the linking sequence comprises GGGGSGGGGSGGGGS (SEQ ID NO: 16)

Embodiment 45. The conjugate of any preceding embodiment, wherein m is 4 to 6; and Y-B is any one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 or SEQ ID NO: 7.

Embodiment 46. The conjugate of any preceding embodiment, wherein the conjugate facilitates internalization of anti-TSHR autoantibody into human cells.

Embodiment 47. The conjugate of any preceding embodiment, wherein the conjugate facilitates accumulation of anti-TSHR autoantibody in an acidic intracellular compartment of human cells.

Embodiment 48. The conjugate of any preceding claim, wherein the conjugate facilitates degradation of anti-TSHR autoantibody by lysosome in the human cells.

Embodiment 49. A method of reducing levels of an extracellular anti-TSHR autoantibody in a patient, the method comprising administering an effective amount of a conjugate according to any one of claims 1 to 45.

Embodiment 50. The method of claim 49, wherein the administration increases internalization of anti-TSHR autoantibody.

Embodiment 51. The method of claim 50, wherein the internalized anti-TSHR autoantibody accumulates into an acidified endosome compartment.

Embodiment 52. The method of any one of claim 49-51, wherein the administration facilitates degradation of the anti-TSHR autoantibody.

Embodiment 53. The method of any one of claim 49-51, wherein the administration results in decrease of greater than 80% of extracellular anti-TSHR autoantibody level in the patient.

Embodiment 54. The method of any one of claims 49-53, wherein the anti-TSHR autoantibody is M22 or K1-18.

Embodiment 55. A method of treating Graves' disease in a patient in need thereof, the method comprising administering to the subject an effective amount of a conjugate according to any one of claims 1 to 45.

Embodiment 56. The method of claim 55, wherein the administration increases internalization of anti-TSHR autoantibody.

Embodiment 57. The method of claim 56, wherein the internalized anti-TSHR autoantibody accumulates in an acidified endosome compartment.

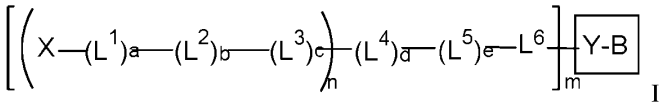
Embodiment 58. The method of any one of claim 54-57, wherein the administration facilitates degradation of the anti-TSHR autoantibody.

Embodiment 59. The method of any one of claim 55-58, wherein the administration results in decrease of greater than 80% of extracellular anti-TSHR autoantibody level in the patient.

Embodiment 60. The method of claims 55-59, wherein the anti-TSHR autoantibody is M22 or K1-18.

WHAT IS CLAIMED IS:

1. A conjugate of Formula I:



I

or a prodrug thereof, or a salt thereof, wherein:

n is 1 to 10;

m is 1 to 10;

X is a moiety that binds to asialoglycoprotein receptor (ASGPR);

Y is a carrier polypeptide connected to B;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody;

each L^1 to L^6 is independently a linking moiety which together provide a linear or branched linker between each X and the Y-B complex; and

a, b, c, d, and e are each independently 1, 2, 3, 4, or 5.

2. The conjugate of claim 1, wherein B comprises an extracellular domain of TSHR or a fragment thereof.

3. The conjugate of claim 1 or 2, wherein B comprises a polypeptide having at least 80% (e.g., at least 85%, or at least 90%) sequence identity with a sequence of SEQ ID NOs: 1-7.

4. The conjugate of any one of claims 1-3, wherein B comprises a polypeptide having at least 95% sequence identity with a sequence of SEQ ID NOs: 1-7.

5. The conjugate of any one of claims 1-4, wherein B comprises a polypeptide having at least 98% sequence identity with a sequence of SEQ ID NOs: 1-7.

6. The conjugate of any one of claims 1-5, wherein B comprises a polypeptide of one of SEQ ID NOs: 1-7.

7. The conjugate of any one of claims 1-6, wherein B consists essentially of a polypeptide of one of SEQ ID NOs: 1-7.

8. The conjugate of claim 1, wherein B has one or more amino acid substitutions selected from R112P, D143P, D151E, V169R, C176S, K250Q, and I253R as compared to a sequence of SEQ ID NO: 1-7.

9. The conjugate of claim 1, wherein B has one or more amino acid substitutions selected from H63C, R112P, D143P, D151E, V169R, I253R as compared to a sequence of SEQ ID NO: 1-7.

10. The conjugate of any one of claims 1 to 9, wherein Y is selected from albumin, albumin binding domain, multimerization domain, Fc domain, Fc (monomer), Fc (dimer), fragments thereof (e.g., synthetic peptides), and variants thereof.

11. The conjugate of any one of claims 1 to 10, wherein Y comprises an albumin, a fragment thereof, or a variant thereof.

12. The conjugate of claim 11, wherein Y comprises human serum albumin (HSA), an HSA domain, bovine serum albumin (BSA), a fragment thereof, or a variant thereof.

13. The conjugate of claim 12, wherein Y is an HSA variant engineered for increased stability, conjugation efficiency (Cys or Lys), and/or FcRn binding.

14. The conjugate of claim 12, wherein Y comprises a polypeptide having 1 to 10 amino acid substitutions, deletions or additions (for example, 1, 2, 3, 4 or 5 amino acid substitutions, deletions or additions) as compared to a sequence of SEQ ID NOs: 8-12, 14 and 15.

15. The conjugate of claim 14, wherein the 1 to 10 amino acid substitutions, deletions or additions are selected from C34A, V54C, K93C, H128C, K262C, and E294C.

16. The conjugate of claim 12, wherein Y comprises a site-specific mutation of a naturally occurring amino acid residue.

17. The conjugate of claim 12, wherein Y comprises a polypeptide having at least 80% (e.g., at least 90%, at least 95%, at least 98%) sequence identity with a sequence of SEQ ID NOs: 8-12, 14 and 15.

18. The conjugate of claim 12, wherein Y comprises a polypeptide of one of SEQ ID NOs: 8-12, 14 and 15.

19. The conjugate of any one of claims 1 to 10, wherein Y comprises a Fc domain, a fragment thereof, or a variant thereof.

20. The conjugate of claim 19, wherein Y comprises Fc (monomer).

21. The conjugate of claim 19, wherein Y comprises Fc (dimer).

22. The conjugate of any one of claims 19 to 21, wherein Y comprises a Fc domain engineered for increased stability, conjugation efficiency (Cys or Lys), and/or FcRn binding.

23. The conjugate of claim 19, wherein Y comprises a polypeptide having 1 to 10 amino acid substitutions, deletions or additions (for example, 1, 2, 3, 4 or 5 amino acid substitutions, deletions or additions) as compared to a sequence of SEQ ID NO: 15.

24. The conjugate of claim 23, wherein the 1 to 10 amino acid substitutions, deletions or additions are selected from T366W, T366S, X368A, A378C, X407V, and L443C.

25. The conjugate of claim 19, wherein Y comprises a polypeptide having at least 80% (e.g., at least 90%, at least 95%, at least 98%) sequence identity with a sequence of SEQ ID NO: 15.

26. The conjugate of claim 19, wherein Y comprises a polypeptide of SEQ ID NO: 15.

27. The conjugate of any one of claims 1 to 26, wherein Y-B is a chimeric fusion protein.

28. The conjugate of claim 27, wherein Y-B is selected from an Fc-TSHR fusion, and an HSA-TSHR fusion.

29. The conjugate of claim 27 or 28, wherein Y is covalently linked to B via a linker.

30. The conjugate of claim 29, wherein the linker comprises a non-peptidic linking moiety.

31. The conjugate of claim 27, wherein Y is fused directly to B.

32. The conjugate of claim 31, wherein Y is fused indirectly to B via a spacer domain.

33. The conjugate of claim 31, wherein the N-terminal of Y is fused to the C-terminal of B.

34. The conjugate of claim 31, wherein the C-terminal of Y is fused to the N-terminal of B.

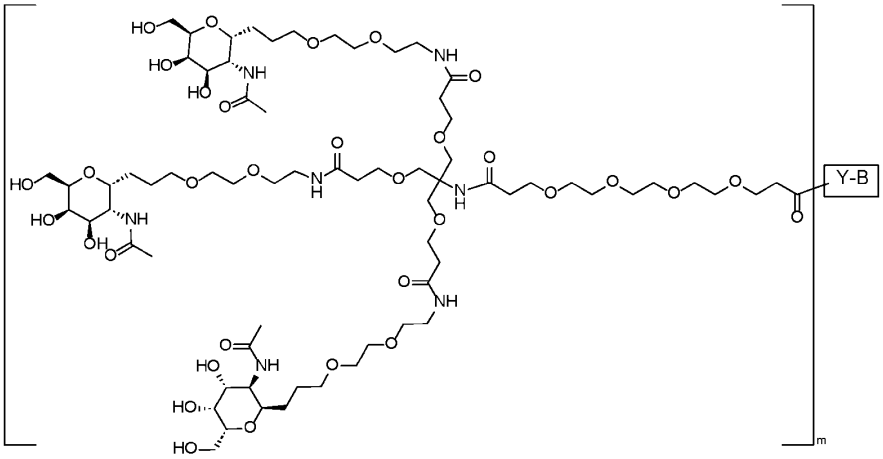
35. The conjugate of any one of claims 1 to 26, wherein Y is covalently linked to B via a non-peptidic linking moiety (e.g., a bifunctional linker).

36. The conjugate of any one of claims 1-35, wherein n is from about 2 to about 10.

37. The conjugate of any one of claims 1-36, wherein L is conjugated to one or more lysine residues of Y or B.

38. The conjugate of any one of claims 1-36, wherein L is conjugated to one or more cysteine residues of Y.

39. A conjugate of Formula:



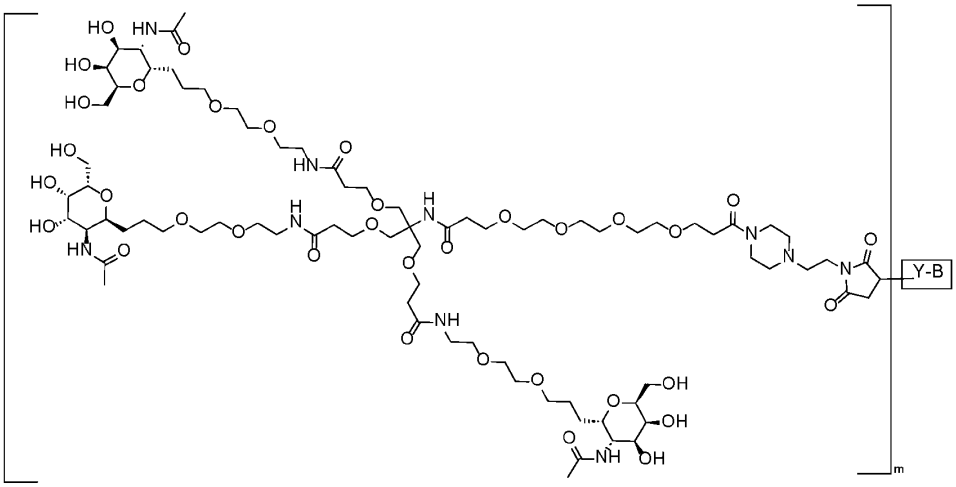
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6.

40. A conjugate of Formula:



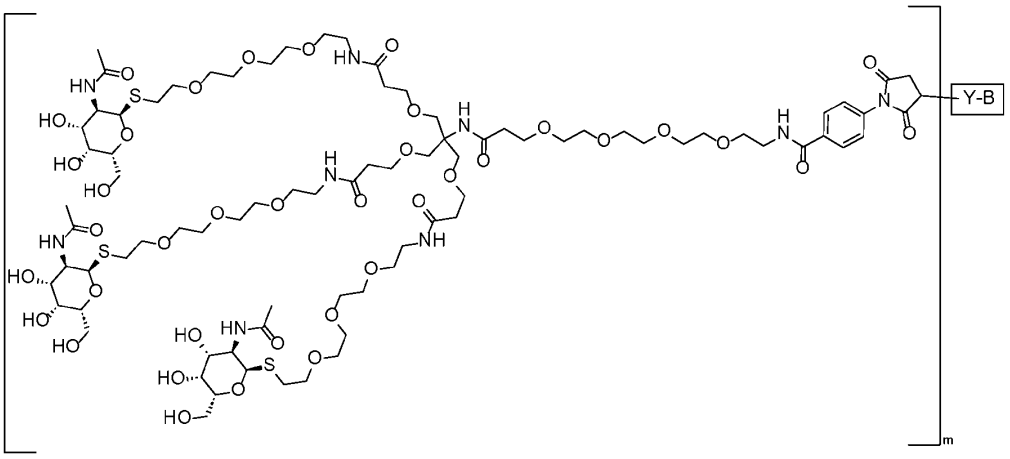
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6.

41. A conjugate of Formula:



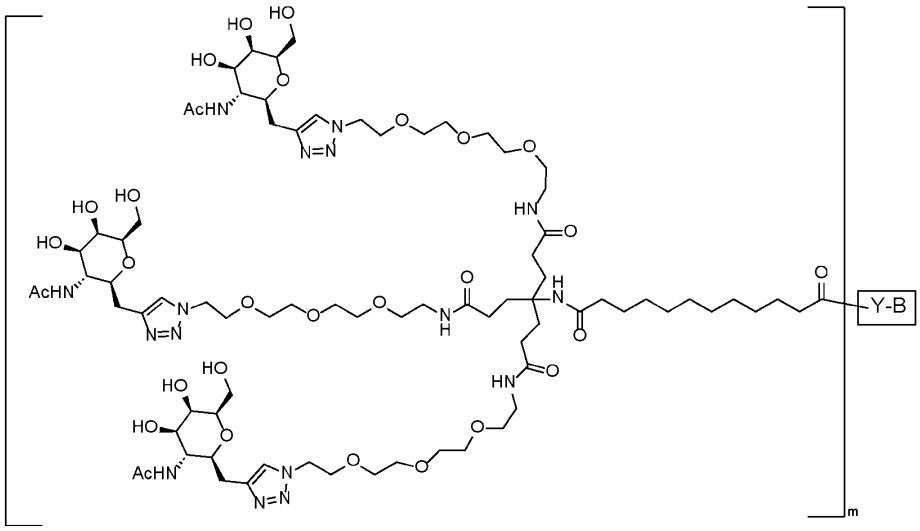
wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6.

42. A conjugate of Formula:



wherein:

m is 4 to 6;

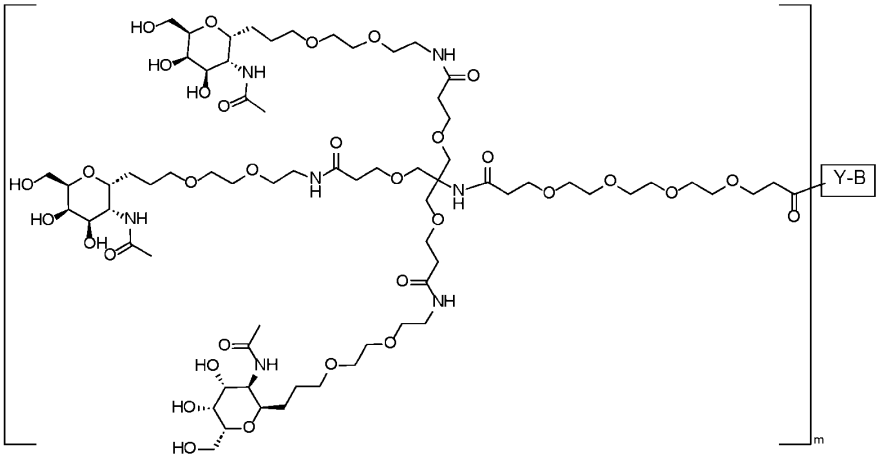
Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15; and

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6.

43. The conjugate of any of claims 39-42, wherein Y is connected to B via a linking sequence.

44. The conjugate of claim 43, wherein the linking sequence comprises GGGGSGGGGSGGGGS (SEQ ID NO: 16).

45. A conjugate of Formula:



wherein:

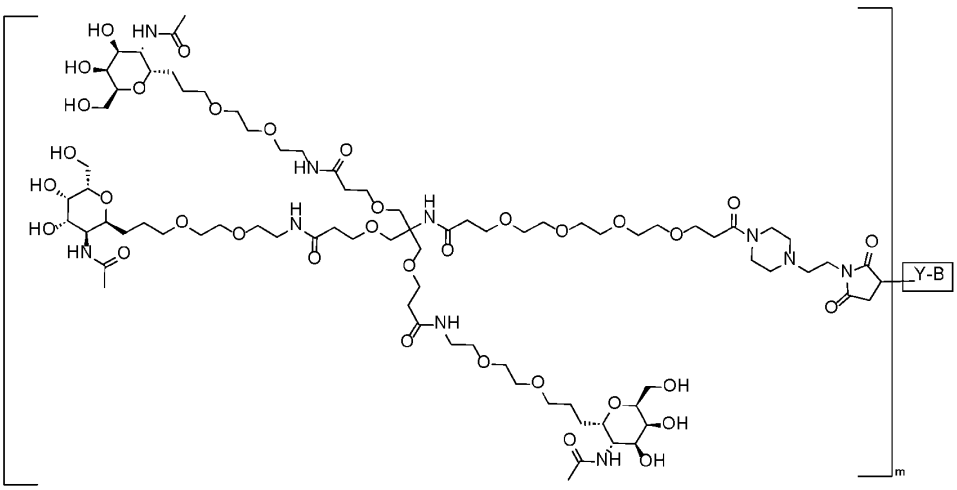
m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15 ;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

46. A conjugate of Formula:



wherein:

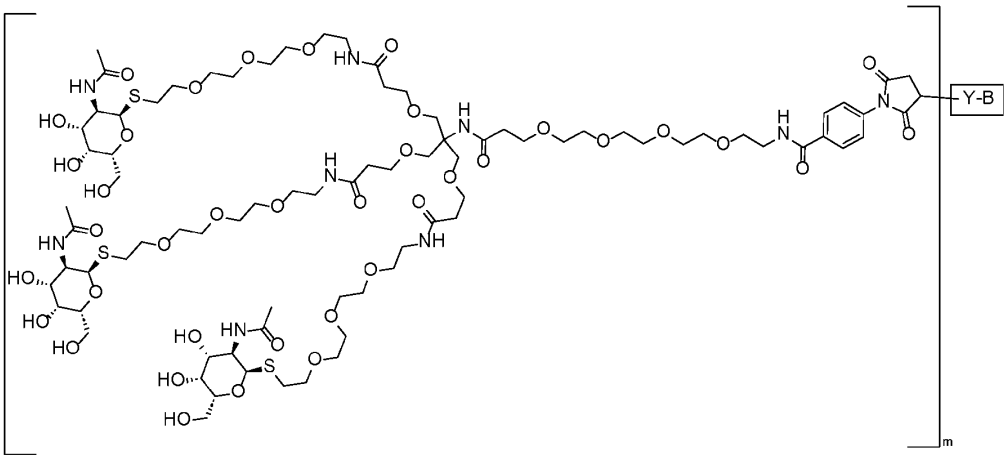
m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

47. A conjugate of Formula:



wherein:

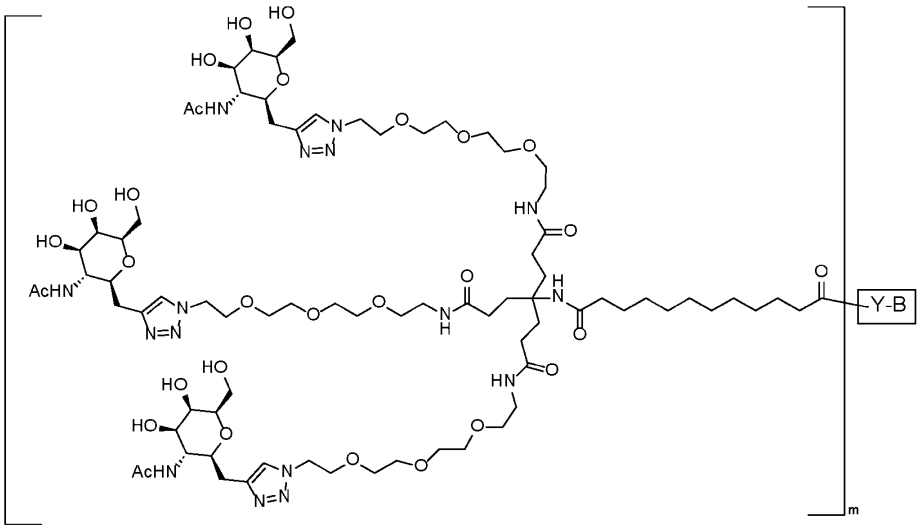
m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

48. A conjugate of Formula:



wherein:

m is 4 to 6;

Y is a carrier polypeptide connected to B, wherein the carrier polypeptide is HSA comprising SEQ ID NO: 15;

B is a thyroid-stimulating hormone receptor (TSHR) polypeptide that binds anti-TSHR autoantibody, comprising SEQ ID NO: 6; and

Y is connected to B via a linking sequence comprising GGGGSGGGGSGGGGS (SEQ ID NO: 16).

49. The conjugate of any preceding claim, wherein the conjugate facilitates internalization of anti-TSHR autoantibody into human cells.

50. The conjugate of any preceding claim, wherein the conjugate facilitates accumulation of anti-TSHR autoantibody in an acidic intracellular compartment of human cells.

51. The conjugate of any preceding claim, wherein the conjugate facilitates degradation of anti-TSHR autoantibody by lysosome in the human cells.

52. A method of reducing levels of an extracellular anti-TSHR autoantibody in a patient, the method comprising administering an effective amount of a conjugate according to any one of claims 1 to 45.

53. The method of claim 52, wherein the administration increases internalization of anti-TSHR autoantibody.

54. The method of claim 53, wherein the internalized anti-TSHR autoantibody accumulates into an acidified endosome compartment.

55. The method of any one of claim 52-54, wherein the administration facilitates degradation of the anti-TSHR autoantibody.

56. The method of any one of claim 52-54, wherein the administration results in decrease of greater than 80% of extracellular anti-TSHR autoantibody level in the patient.

57. The method of any one of claims 52-56, wherein the anti-TSHR autoantibody is M22 or K1-18.

58. A method of treating Graves' disease in a patient in need thereof, the method comprising administering to the subject an effective amount of a conjugate according to any one of claims 1 to 45.

59. The method of claim 58, wherein the administration increases internalization of anti-TSHR autoantibody.

60. The method of claim 59, wherein the internalized anti-TSHR autoantibody accumulates in an acidified endosome compartment.

61. The method of any one of claim 58-60, wherein the administration facilitates degradation of the anti-TSHR autoantibody.

62. The method of any one of claim 58-61, wherein the administration results in decrease of greater than 80% of extracellular anti-TSHR autoantibody level in the patient.

63. The method of claims 58-52, wherein the anti-TSHR autoantibody is M22 or K1-18.