

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(10) International Publication Number
WO 2021/113851 A2

(43) International Publication Date
10 June 2021 (10.06.2021)

(51) International Patent Classification:

C12N 15/113 (2010.01)

(21) International Application Number:

PCT/US2020/063676

(22) International Filing Date:

07 December 2020 (07.12.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/945,042 06 December 2019 (06.12.2019) US

(71) Applicant: **SIRNAOMICS, INC.** [US/US]; 401 Professional Drive, Suite 280, Gaithersburg, MD 20879 (US).

(72) Inventors: **LU, Xiaoyong**; c/o Sirnaomics, Inc., 401 Professional Drive, Suite 280, Gaithersburg, MD 20879 (US). **LU, Patrick, Y.**; c/o Sirnaomics, Inc., 401 Professional Drive, Suite 280, Gaithersburg, MD 20879 (US). **EVANS, David, M.**; c/o Sirnaomics, Inc., 401 Professional Drive, Suite 280, Gaithersburg, MD 20879 (US).

(74) Agent: **BOOTH, Paul M.** et al.; Edell, Shapiro & Finnan, LLC, 9801 Washingtonian Blvd., Suite 750, Gaithersburg, MD 20878 (US).

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: PEPTIDE DOCKING VEHICLE FOR TARGETED NUCLEIC ACID DELIVERY

(57) Abstract: Peptide docking vehicle compositions containing a therapeutic compound, such as an siRNA molecule, and a targeting ligand are provided, together with methods for their preparation and use. The compositions and methods allow targeted cell/tissue delivery of the therapeutic compound to a subject by linking a targeting ligand to the compound to provide enhanced therapeutic benefit. The subject may be an animal or a human.



WO 2021/113851 A2

PEPTIDE DOCKING VEHICLE FOR TARGETED NUCLEIC ACID DELIVERY**FIELD OF THE INVENTION**

Delivery systems are provided in which single or multi-nucleic acids are covalently
5 conjugated to an endosomal releasing peptide, and are further covalently linked to at least one
small molecular ligand. Novel compositions of matter are provided, together with methods
for their preparation and use. With the selected ligand and linker, the compositions and
systems provide targeted delivery of nucleic acid molecules to the liver and other tissue.

BACKGROUND

10 Double-stranded RNA has been shown to silence the gene expression (Fire et. al.,
Nature 391:806–811 (1998), and Elbashir et. al., *Nature*, 411, 494–498 (2001)) and this
phenomenon has been named RNA interference or “RNAi”, or interfering RNA molecule
(RNAi). Short-interfering RNA (siRNA) induced RNAi regulation shows great potential to
treat a wide variety of human diseases from cancer to other traditional undruggable diseases.
15 There are three major advantages of RNA-based therapeutics over traditional small-molecule
and antibody therapeutics. First, once delivery to a specific cell type or tissue has been
devised (e.g., siRNA/anti-sense oligonucleotide (ASO) delivery to hepatocytes; ASO delivery
to the CNS), it is likely that every disease-promoting gene in that cell type can be targeted.
Second, RNA therapeutics can selectively target single genes and can be readily engineered
20 to avoid regulating off-target genes, whereas small-molecule inhibitors often hit multiple
targets and have unknown off-target side effect. Third, unlike static small molecules and
antibodies, RNA therapeutics can pharmacoevolve their sequence to keep pace with, for
example, cancer mutations or pandemic influenza. These attributes provide RNA-based
therapeutics with great potential to treat undruggable human diseases, provided delivery can
25 be properly solved.

In the field, ASO, RNAi and mRNA chemistry has advanced to enable the improved
stability and avoid the innate immune response. It also allows industrial scale production of
therapeutic ASO and RNA compounds on a large scale via automated synthesis at relatively
low cost compared to antibody or small molecule development. However, two areas still need
30 further development: (a) targeting of these molecules to specific cell types or tissues; and (b)
the devising of non-toxic endosomal escape agents. Dowdy, *Nature Biotechnology*, 35:222-
229 (2017).

Two types of effective delivery method are used in marketed ASO or RNAi drugs. One uses lipid based nanoparticle called liposomes that contain multiple components. The other one uses a GalNAc molecule-conjugated ASGPR-targeted (asialoglycoprotein receptor) approach.

5 A major challenge for RNA-based therapeutics is that all pathways for delivery to cells eventually lead to endosomal escape. ASO and siRNA delivery to the liver can be achieved using ASGPR-targeted GalNAc-siRNA conjugates due to the properties of ASGPR that are well suited for macromolecular drug deliver to hepatocytes. In particular, hepatocytes express millions of copies of ASGPR on their cell surface, which cycle at a rapid rate of
10 every 10–15 min. These properties make a GalNAc-based delivery approach effective even with a presumed endosomal escape rate of <0.01%. By contrast, effective delivery of ASO or RNA to other tissues has not been achieved. No other ligand–receptor system expresses receptors at such a high level as ASGPR, nor cycles into endosomes as rapidly. Indeed, most cell surface receptors are expressed in the range of 10,000–100,000 per cell (or lower), and
15 caveolin and clathrin-mediated endocytosis typically recycles every 90 min. See Juliano, *Nucleic Acids Res.* 44, 6518–6548 (2016).

Endosomal escape remains a problem that applies to all RNA-based therapeutics. Enhancing endosomal escape by developing new chemistries and materials is needed to target the cell or tissue beyond the liver hepatocytes. Small-molecule endosomolytic agents such as
20 chloroquine have been used to disrupt or lyse endosomes, but at the effective concentration these agents invariably lyse all types of endosomes inside the cell resulting in substantial toxicity.

An alternative endosomal escape approach is to conjugate endosomolytic peptides or molecules directly to the RNA, which will strictly limit their action to endosomes containing
25 the RNA therapeutic. Various clinical trials using a two-molecule dynamic polyconjugate (DPC) system containing cholesterol or lytic melittin peptide to escape the endosome were terminated due to toxicity effects. Wooddell, *et al.*, *Mol. Ther.* 21, 973–985 (2013); Hou *et al.*, *Biotechnol. Adv.* 33, 931–940 (2015).

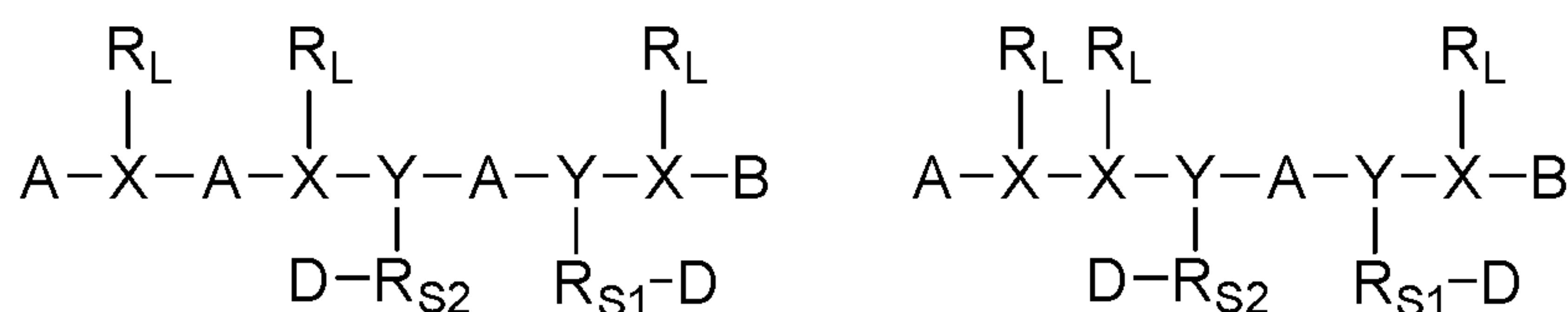
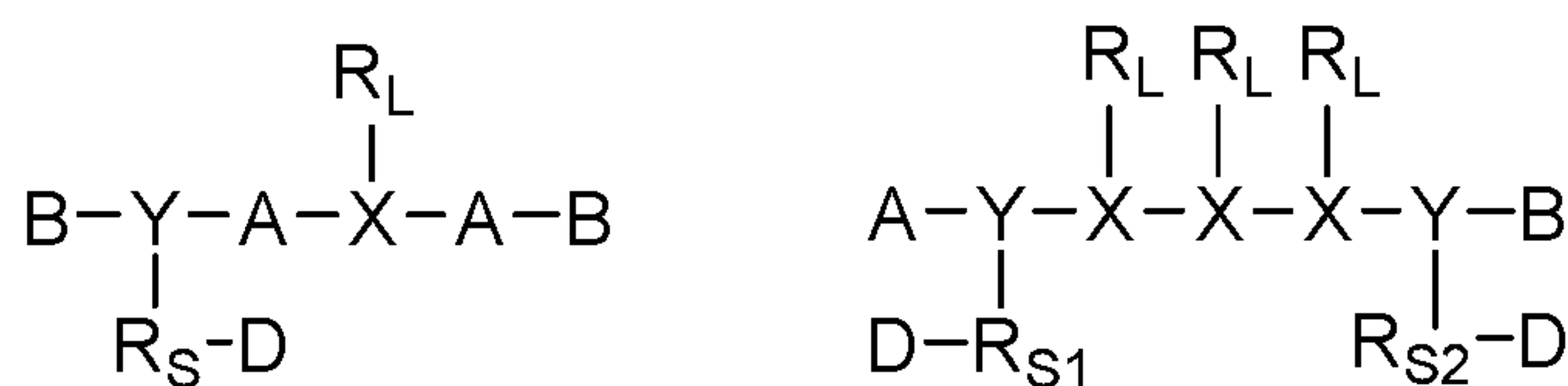
The compositions and methods described below use endosomal release peptides
30 conjugated to oligonucleotides and targeting ligands. The constructs enhance escape of the oligonucleotide cargo into the cytoplasm of cells in a non-toxic manner. The construct of the invention is referred to herein as a “Peptide Docking Vehicle” (PDoV).

SUMMARY OF THE INVENTION

What is provided is a chemical construct containing a Peptide Docking Vehicle (PDoV) covalently linked to (a) a targeting moiety, and (b) a first therapeutic oligonucleotide. The construct may contain a second therapeutic oligonucleotide that is the same as, or
 5 different from, the first therapeutic oligonucleotide. The PDoV may contain multiple repeating units of histidine and lysine. In one embodiment the targeting moiety binds to the asialoglycoprotein receptor. The oligonucleotide may contain an siRNA, an antisense oligonucleotide, an miRNA, an saRNA, an shRNA, an aptamer, an RNAzyme, an DNAzyme, a decoy oligonucleotide, or a CpG motif.

10 In these constructs, the PDoV construct may contain an endosomal release motif that may contain at least two targeting moieties that are the same or different, and/or at least two therapeutic oligonucleotides that are the same or different.

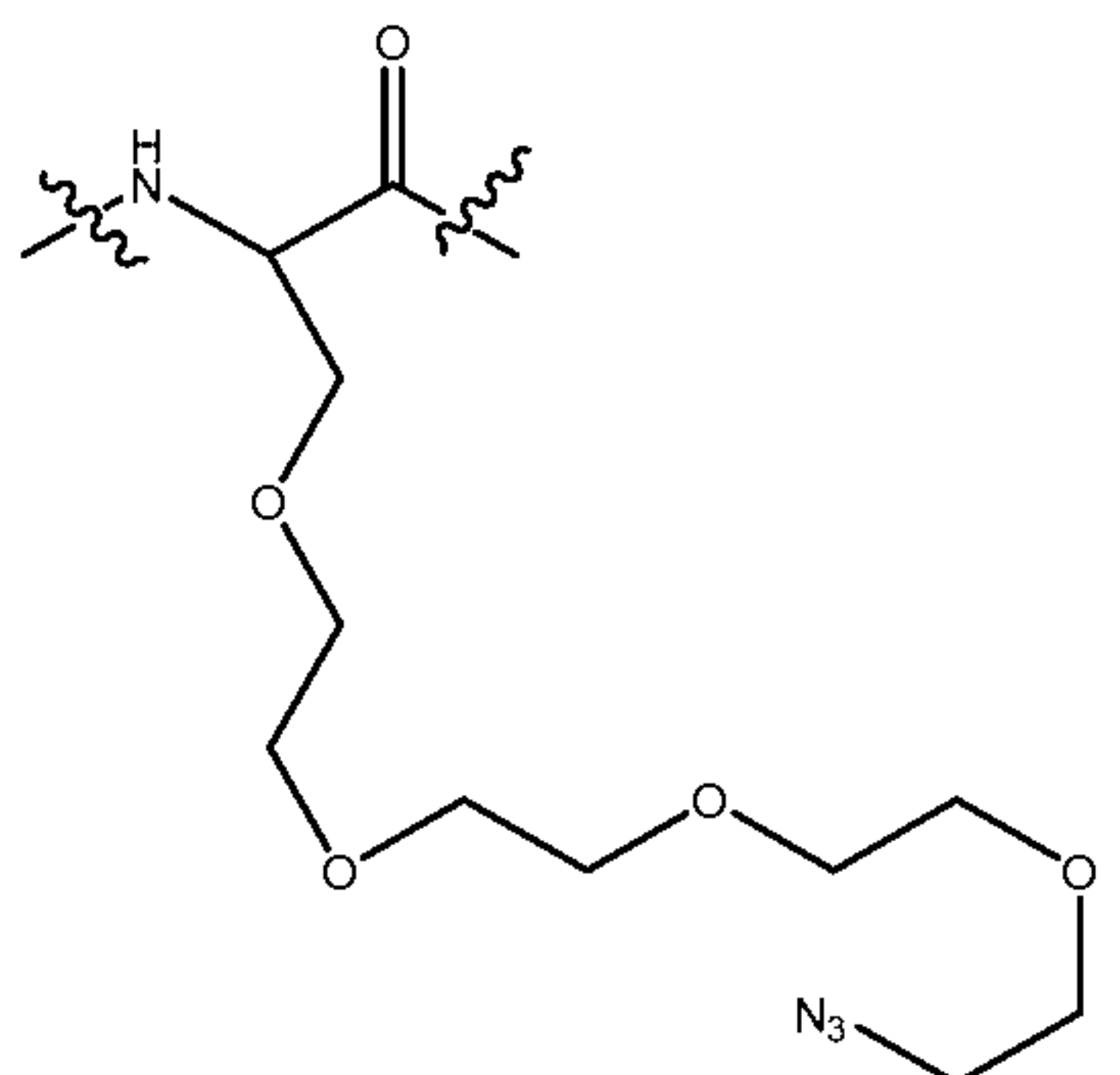
The construct may have one of the structures shown below, where: type X sites are used to conjugate the targeting ligands; Type Y sites are used to conjugate the
 15 oligonucleotide, and X and Y can be the same or different; A is a peptide sequence of H, K, R, HH, HHH, HHHH, HHK, HHHK or any other endosomal releasing short peptide containing up to about 5 amino acids; B is a peptide sequence of H, K, R, HH, HHH, HHHH, HHK, HHHK, or any other short peptide containing up to about 5 amino acids, any other amino acid or combination of amino acids with a linker; D is an oligonucleotide; R_L is ligand;
 20 and R_S is a linker to the oligonucleotide.



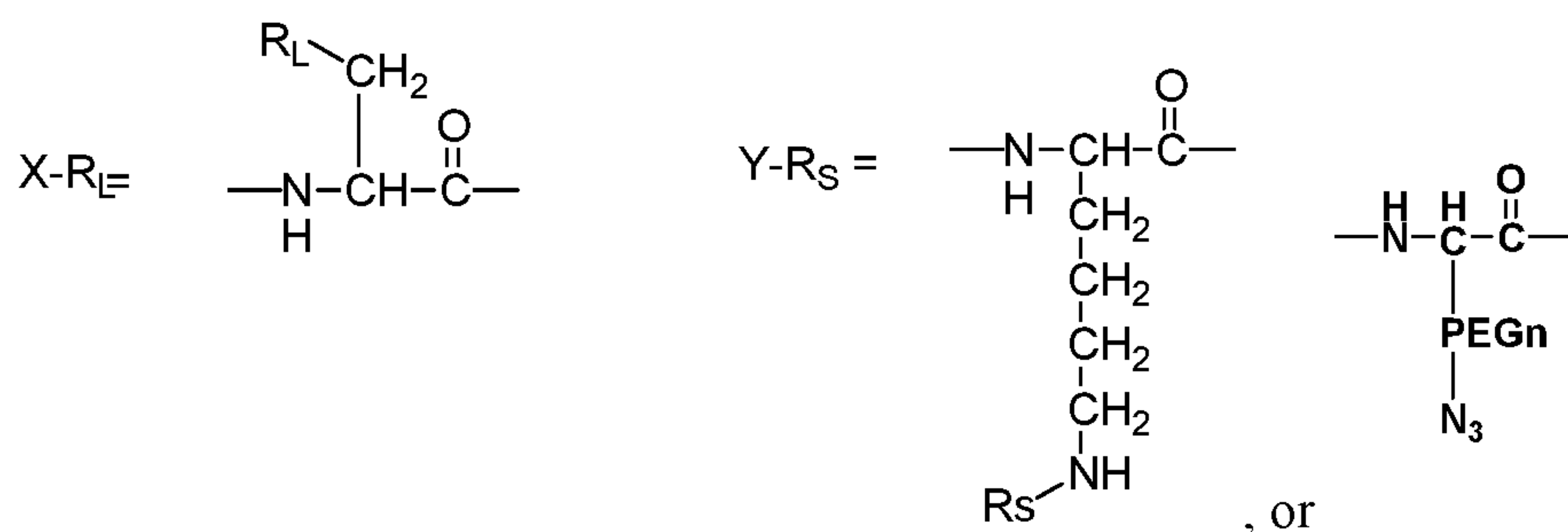
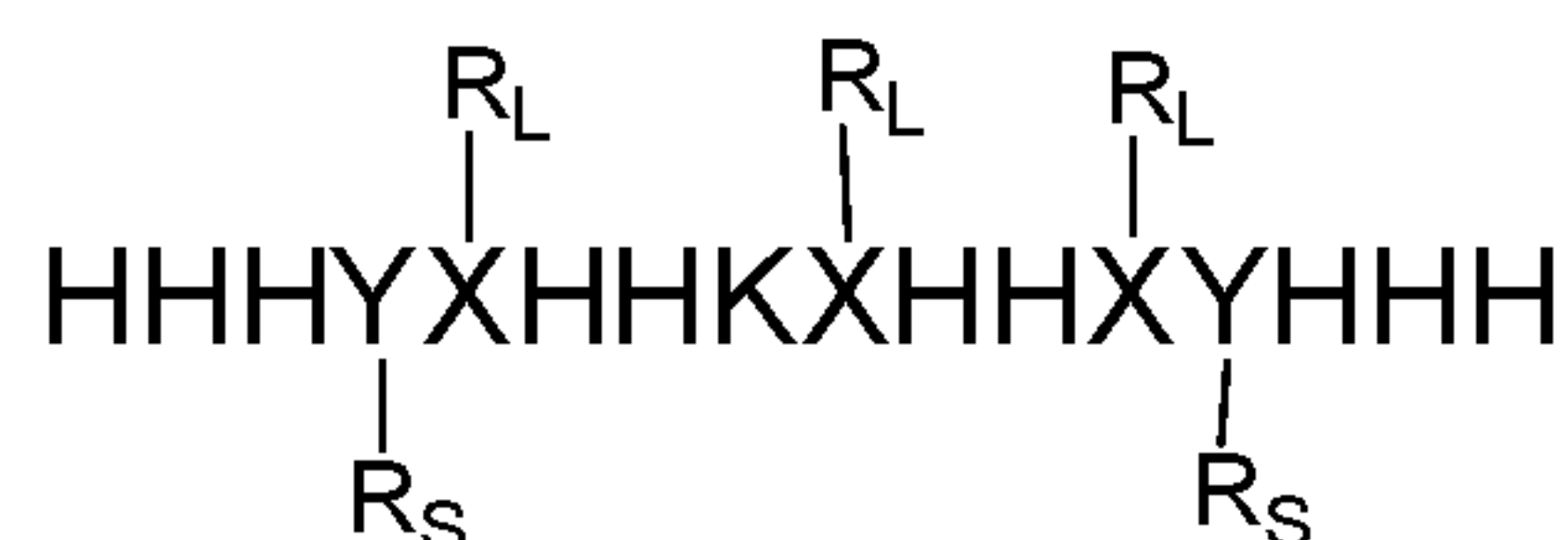
D may be an siRNA, mRNA, or an aptamer.

In these constructs, the PDoV peptide construct may have a structure containing an amino acid sequence selected from the group consisting of KHHHCKH, HKHHHCKH,
 25 HHKHHHCKH, HHHKHHHKCHHHKHHH, HHHKHHCKHHH, HHHKHHCRHHH, HKHHCKH, HKHCH, HKHCKH, HKHC, HHHK(S)HHCKHHH, and

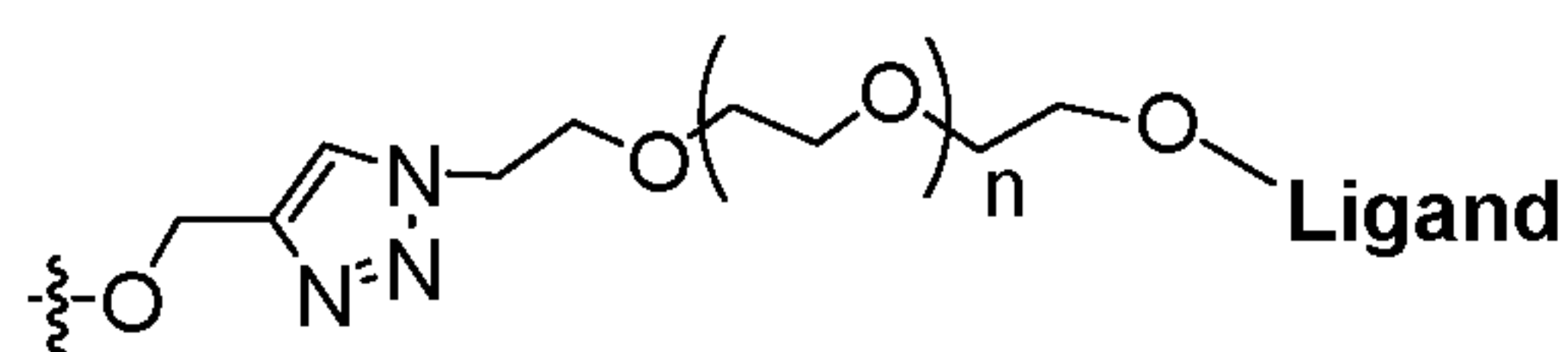
HHK(S)HHKCHH(S)HHH, where R_L is linked to the side chain of the cysteine residue and R_S is linked to the side chain of the lysine residue, and where (S) is:



- 5 In other embodiments, the PDoV peptide construct may have the structure below, in which three of the ligands are conjugated individually on the peptide.

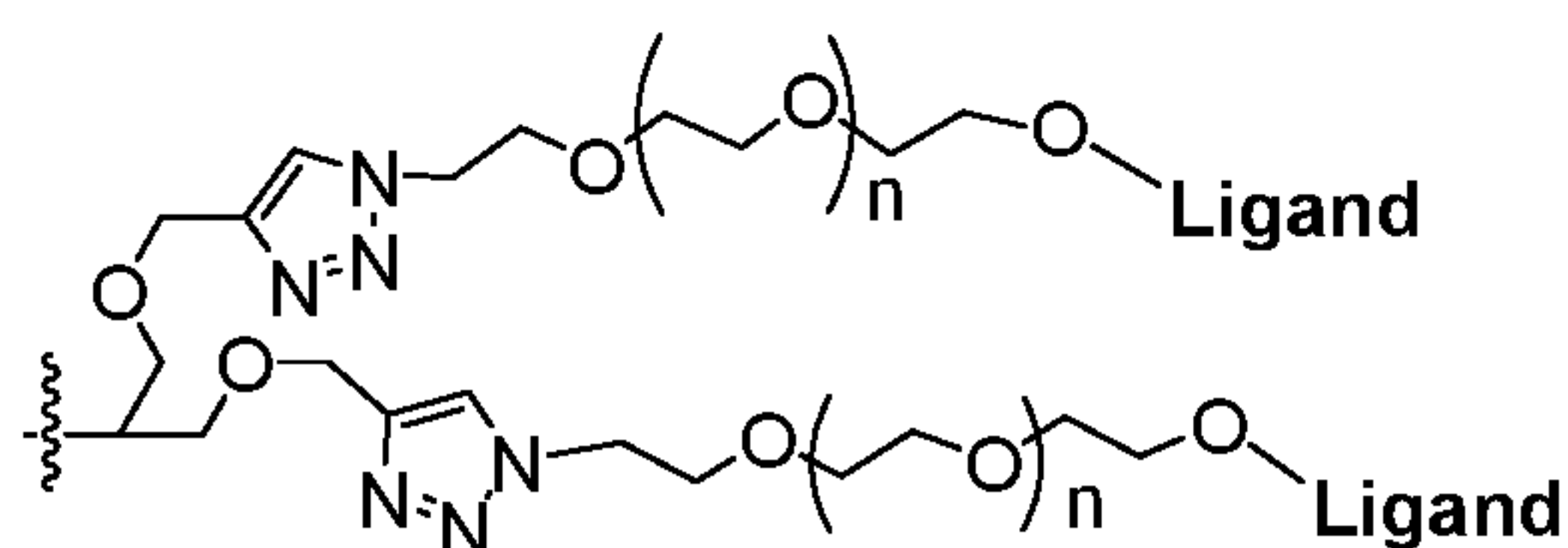


The ligand moiety R_L may contain linker-1, where linker-1 may contain one of the following structures:



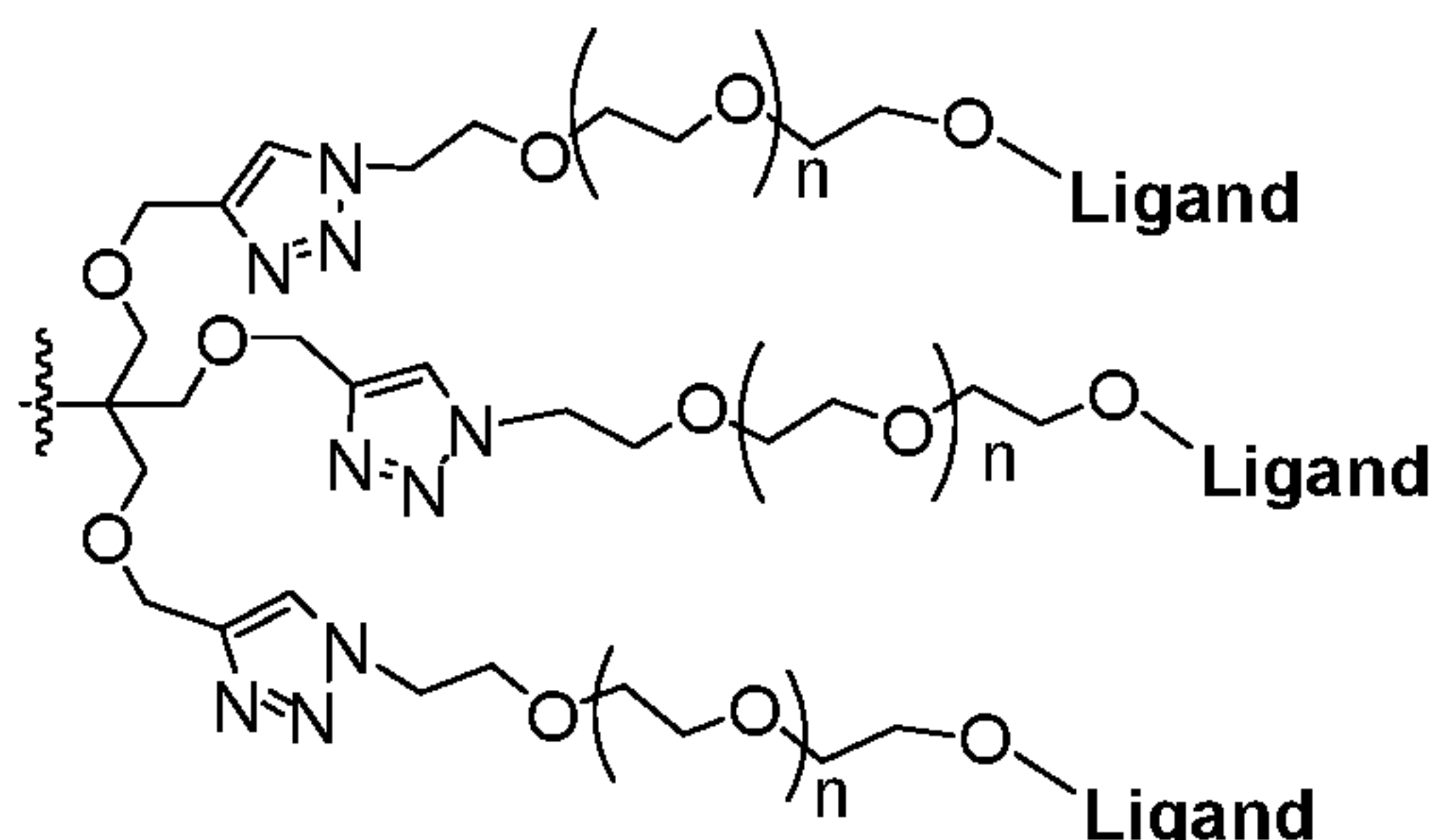
10

where n is 1, 2, or 3 and is connected to the bridge through a 1, 5-triazol ring with an



OCH₂ unit; or

where n is 1, 2, or 3 and is connected to the bridge through a 1, 5-triazol ring with a CH₂OCH₂ unit; or

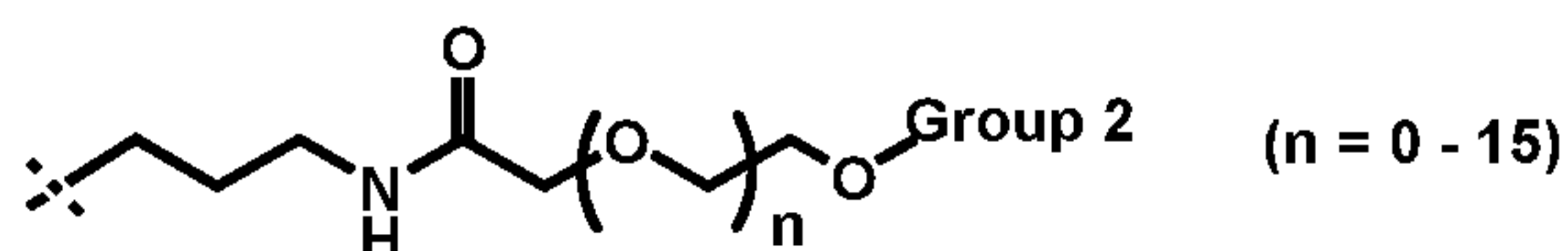
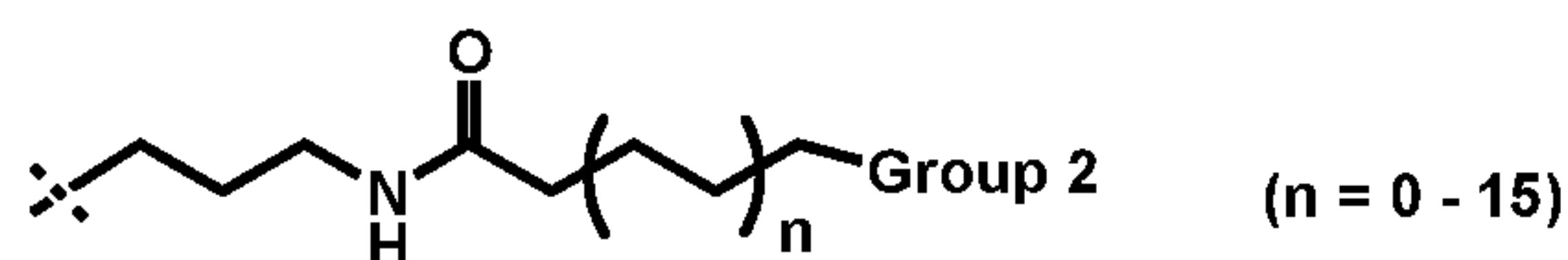


where n is 1, 2, or 3 and is connected to the bridge through a 1, 5-triazol ring with a CH₂OCH₂ unit.

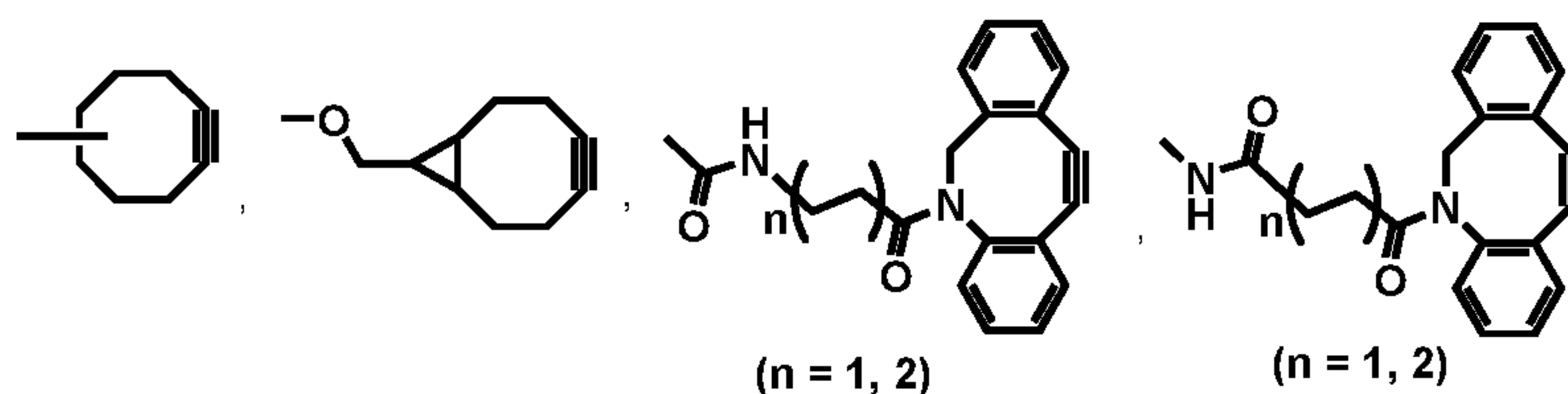
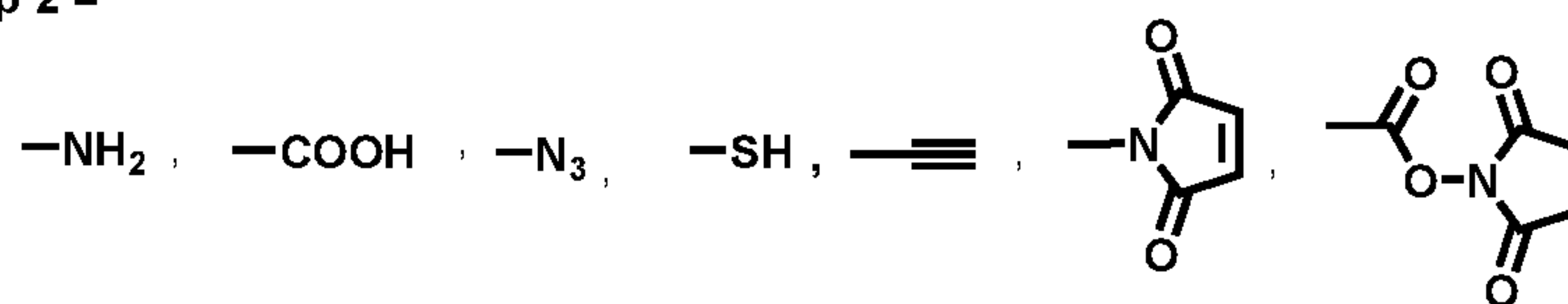
The linker between the ligand(s) attached to the PDoV peptide may contain a polyethylene glycol chain $-(CH_2CH_2O)_n-$, or $-(CH_2CH_2)_n-$ chain, where n is an integer from 2-15. The ligand R_L may be linked to the PDoV peptide via a linker that may contain a thioether, an amide, or a triazole linkage.

In other embodiments the oligonucleotide is linked via a moiety having the structure Rs-linker2-oligonucleotide, where linker 2 is an aliphatic chain, a polyethylene glycol chain, a hydrophobic lipid chain or a hydrophilic chain. The group 2 at the end site is the reactive site for the chemical conjugation with the siRNA end.

Linker 2 =

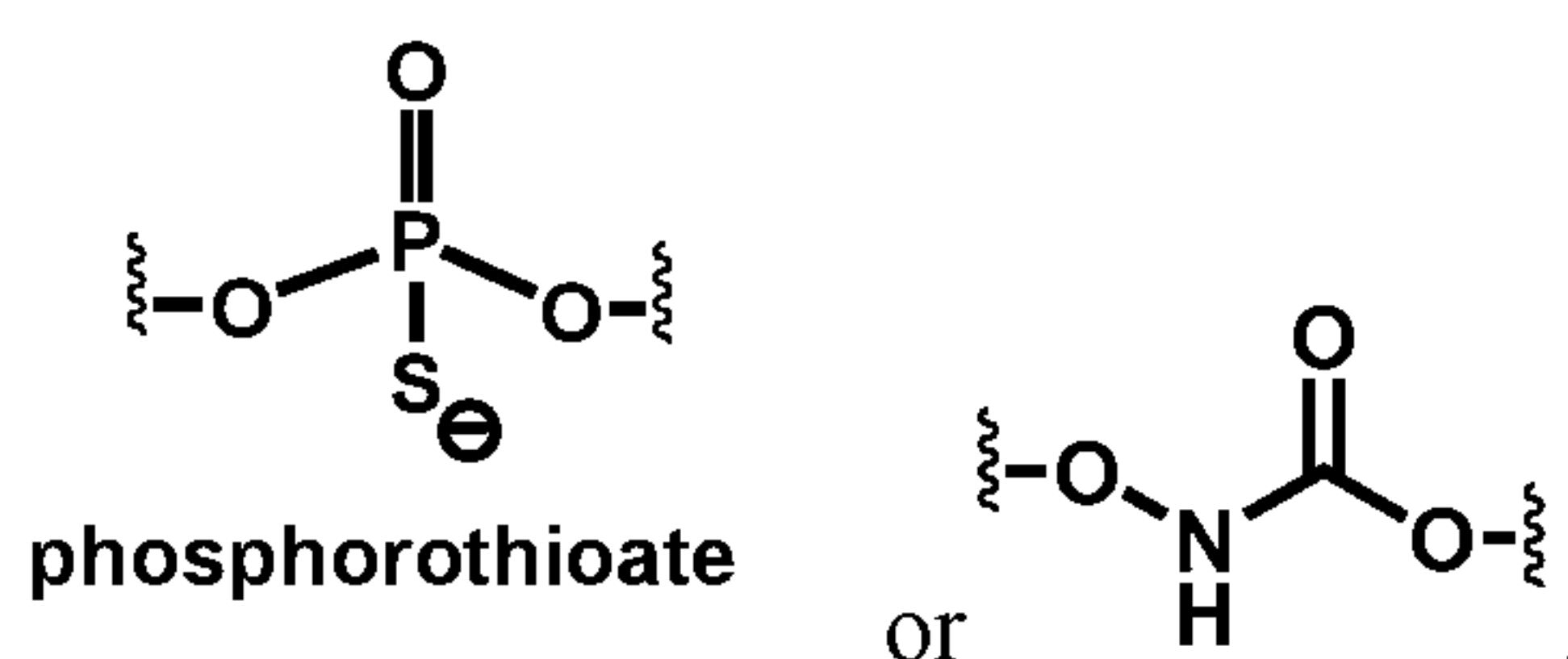


Group 2 =



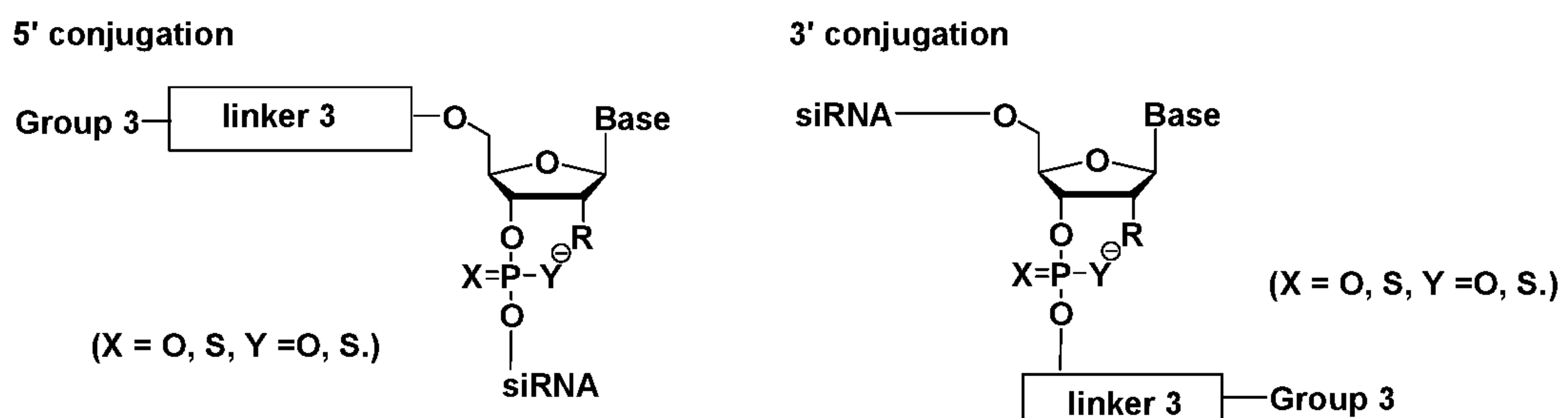
The oligonucleotide may be an RNAi molecule that is single-stranded oligonucleotide with a length of 10-29, or may be a duplex of two complimentary, single-stranded oligonucleotides each with a length between 10-29 bases or 19-27 bases. oligonucleotide may contain deoxyribonucleotides and/or ribonucleotides. The oligonucleotide may contain an siRNA molecule containing at least one or more chemically modified nucleotides at the 2' position, where, for example, the chemically modified nucleotides comprise 2'-O-Methyl or 2'-Fluoro, 2'-O-methoxyethyl, 2'-O-allyl, or 2'-H, modified nucleotides.

In other embodiments, the oligonucleotide may contain an siRNA molecule containing at least one chemically modified linkage that is



In other embodiments, the siRNA or oligonucleotide is further chemically modified at position 5' or 3' with a linker 3, where Linker 3 contains a complimentary conjugation site group 3 which can covalently react with the linker 2 to link the ligand-PDoV and nucleotides.

15 In further embodiments, the construct may have a structure as shown below:



In each of these constructs, the targeting ligand may be selected from the group consisting of N-acetyl-galactosamine (GalNAc), galactose, galactosamine, N-formal-galactosamine, N-propionyl-galactosamine, N-butanoylgalactosamine and aptamer. In other embodiments the targeting ligand is a peptide selected from the group consisting of cyclic(c) RGD, APRPG, NGR, F3 peptide, CGKRR, LyP-1, iRGD (CRGDRCPDC), iNGR, T7 peptide (HAIYPRH), MMP2-cleavable octapeptide (GPLGIAGQ), CP15 (VHLGYAT), FSH (FSH-β, 33-53 amino acids, YTRDLVKDPARPKIQKTCTF), LHRH (QHTSYkcLRP),

gastrin-releasing peptides (GRPs) (CGGNHWAVGHLM), RVG (YTWMPENPRPGTPCDIFTNSRGKRASNG), FMDV20 peptide sequence (NAVPNLRGDLQVLAQKVART), and GLP or is folic acid.

In any of these constructs the peptide may contain an amino acid sequence -HHK, -KHH, -HHH, -HHHK, -KHHH, -HHHHK, -KHHHH, -K(HHHK)_n (n= 1-8), -K(HHHHK)_n(HHHK)_m (n= 1-4, m=1-8), K(HHHHK)_n(HHHK)_m- (n= 1-4, m=1-8), for example the construct may contain a peptide with a sequence -K(HHHHK)_n(HHHK)_m, or K(HHHHK)_n(HHHK)_m-, where n is 1 and m is 3. The peptide may be an endosomal releasing peptide or may be a cell penetrating peptide.

Also provided is a peptide with the formula (H_nK_m)_oX_pY_q, where H is histidine, K is lysine, X and Y are functional units selected from an amino acid and a linker, n is 1-10, m is 1-10, o is 1-10, p is 1-5, and q is 1-4. The peptide may include at least two conjugation sites for attaching a targeting ligand. Advantageously the conjugation site is lysine or cysteine. The peptide may contain at least two conjugation sites for attaching an oligonucleotide. The construct may contain the peptide of formula (H_nK_m)_oX_pY_q and at least one targeting ligand linked to the peptide via at least one conjugation site. At least one oligonucleotide may be linked to the peptide via at least one conjugation site. The targeting ligand may be selected from the group consisting of cyclic(c) RGD, APRPG, NGR, F3 peptide, CGKRK, LyP-1, iRGD (CRGDRCPCDC), iNGR, T7 peptide (HAIYPRH), MMP2-cleavable octapeptide (GPLGIAGQ), CP15 (VHLGYAT), FSH (FSH-β, 33-53 amino acids, YTRDLVKDPPARPKIQKTCTF), LHRH (QHSTSYkcLRP), gastrin-releasing peptides (GRPs) (CGGNHWAVGHLM), RVG (YTWMPENPRPGTPCDIFTNSRGKRASNG), FMDV20 peptide sequence (NAVPNLRGDLQVLAQKVART), and GLP. The oligonucleotide may be selected from the group consisting of an siRNA, an antisense oligonucleotide, an miRNA, an saRNA, an shRNA, an aptamer, an RNAzyme, an DNAzyme, a decoy oligonucleotide, and a CpG motif.

Also provided are pharmaceutical compositions containing a construct as described above claim and a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may contain histidine -lysine rich polypeptide HKP, HKP(H), or lipofectamine, and/or may contain water and one or more of the group consisting of: potassium phosphate monobasic anhydrous NF, sodium chloride USP, sodium phosphate dibasic heptahydrate USP, glucose, and Phosphate Buffered Saline (PBS).

Also provided are methods of delivering an oligonucleotide to a cell, which include contacting the cell with a construct or pharmaceutical composition as described above.

Also provided are methods of delivering an oligonucleotide to a hepatocyte cell, which include contacting the cell with a construct as described above.

In these methods, the oligonucleotide may be an siRNA molecule and may be delivered to the mammalian cell or hepatocyte *in vivo*. The cell may be a human cell.

5 Also provided are methods of gene therapy in a mammal by administering a therapeutically effective amount of a construct as described above to the mammal, where the mammal optionally is a human.

Also provided are methods of treating cancer in a mammal, such as a human, including administering to the mammal a therapeutically effective amount of a construct as described above. The cancer may be a solid tumor. The cancer may be liver cancer, colon cancer, or pancreatic cancer. The liver cancer may be hepatocellular carcinoma, metastatic colon cancer, or metastatic pancreatic cancer. The construct may be injected directly into a tumor containing the cancer cells.

BRIEF DESCRIPTION OF THE FIGURES

15 Figure 1 shows a cartoon representation of the design of the [GalNAc] Peptide Docking Vehicle (G-PDoV). Trivalent GalNAc was covalently conjugated on one docking site A. Oligonucleotide or siRNA was conjugated on the other one or two docking sites B respectively.

Figure 2 shows a cartoon representation of an alternative design of a [ligand] Peptide Docking Vehicle. Monovalent GalNAc was covalently conjugated at one to four docking sites A. Oligonucleotide or siRNA were conjugated on the other one or two docking sites B respectively.

Figure 3 shows the design of the Peptide Docking Vehicle (PDoV): it has a $(H_nK_m)_oX_pY_q$ peptide backbone with multiple repeating units of histidine (H), lysine (K) and functional units X and Y (amino acid, or functional linker), wherein $n = 1-10$, $m = 1-10$, $o = 1-10$, $p = 1-5$, $q = 1-5$. HK repeating units have been shown to have good cell penetrating ability, and to facilitate endosome release, the lysine or the various functional unit X or Y will be adopted as the docking sites for the conjugation of ligands and Y will be adopted as the docking sites for the conjugation of oligonucleotide through a different covalent linkage.

25 For example, the site ① will only be able to react in the presence of ligand such as GalNAc or other targeting ligands. The site ③ can only conjugate with oligonucleotide and siRNA under the specific condition..

30

Figure 4 shows the design of a Peptide Docking Vehicle (PDoV) with three ligand conjugation sites and multi oligonucleotide sites: it has a $(H_nK_m)_oX_pY_q$ peptide backbone with multi- repeating units of histidine (H), lysine (K) and functional units X and Y (amino acid, or functional linker), wherein the $n = 1-10$, $m = 1-10$, $o = 1-10$, $p = 1-5$, $q = 1-5$. HK repeating units have been demonstrated to have good cell penetrating ability and to facilitate endosome release. The lysine residues or the various functional units X are adapted as the docking sites for conjugation of ligands, and Y is adapted as the docking site(s) for the conjugation of oligonucleotides through different covalent linkages. For example, the site ① will only be able to react in the presence of a ligand such as GalNAc or other targeting ligands. The site ③ can only conjugate with oligonucleotides and siRNAs under specific conditions.

Figure 5 shows the structure of the PDoV construct. The PDoV construct is a cell penetrating/endosome releasing peptide inserted with multiple conjugation sites X and Y. Site X is used to conjugate the targeting ligand, and site Y is used to conjugate multiple oligonucleotide or nucleic acid. Some construct examples for the PDoV include where: A represents a peptide sequence K, R, H, HH, HHH, HHHH, HHK, HHHK or other short peptide; B represents a peptide sequence K, R, H, HH, HHH, HHHH, HHK, HHHK, or other short peptide, other amino acid or combination; D represents oligonucleotide, siRNA, mRNA, aptamer; RL represents ligand; and RS represents linker to oligonucleotide.

Figure 6 shows an example of the structure of a first generation PDoV, containing one or two oligonucleotide sites and one ligand conjugation site.

Figure 7 shows an example of the structure of a second generation PDoV, containing two oligonucleotide sites and one multivalent ligand conjugation site.

Figure 8 shows an example of alternative structure of a PDoV, containing two oligonucleotide sites and one multivalent ligand conjugation site. The ligands can be conjugated individually one by one on the PDoV backbone.

Figure 9A shows linkage selection for the conjugation sites. Chemical group Rs represents a “click” like reactive moiety to conjugate the oligonucleotide with the PDoV peptide vehicle. The reactive moiety can be amine, hydrazine, N-hydroxysuccinimide, azido, alkyne, carboxylic acid, thiol, maleimide, or other chemical reactive moiety known in the art

Figure 9B shows representative examples of the linkage.

Figure 9C shows how the linker 2 in the conjugation Rs-linker2-siRNA is a chemical spacer between the peptide and the conjugation site, allowing the conjugation site to be

attached at the linker's terminal site. The linker 2 can be an aliphatic chain or a polyethylene glycol chain, or other hydrophobic lipid or hydrophilic chain. The group 2 at the end site is the reactive site for the chemical conjugation with the siRNA end.

Figure 10 shows an example of the linkage selection for the ligand conjugation sites.

5 The linkage for the ligand conjugation R_L can be a selection based on the monovalent GalNAc molecule, bivalent GalNAc molecule and trivalent GalNAc molecule. The conjugation site can be maleimide/thiol or may be selected from the Group 2 list shown in Figure 9.

10 Figure 11 shows a representative example of the construction of the siRNA-PDoV-ligand compound 1

Figure 12 shows a representative example of construction of the siRNA-PDoV-ligand compound 2.

Figure 13 shows a representative example of construction of the siRNA-PDoV-ligand compound 3.

15 Figure 14 shows a list of some possible siRNA sequence and structure modifications.

Figure 15A shows *in vitro* serial dilution data for the conjugate of mTTR1-PDoV3-GalNAc3. Figure 15B shows *in vitro* serial dilution data for the mTTR1-PDoV2-GalNAc3 conjugate. Figure 15C shows serial dilution data for the mTTR2-GalNAc3 positive control conjugate. Figure 15D shows *in vitro* serial dilution data for the Aha1-GalNAc3 positive
20 control conjugate.

DESCRIPTION OF THE INVENTION

Definitions:

As used herein, "oligonucleotide" refers to a chemically modified or unmodified
25 nucleic acid molecule (RNA or DNA) having a length of less than 100 nucleotides (for example less than 50, less than 30, or less than 25 nucleotides). It can be siRNA, microRNA, anti microRNA, microRNA mimics, dsRNA, ssRNA, aptamer, triplex forming oligonucleotides, aptamers. In one embodiment, the oligonucleotide is an RNAi agent.

As used herein, an "siRNA molecule" or "RNAi molecule" is a duplex oligonucleotide, that
30 is a short, double-stranded polynucleotide, that interferes with the expression of a gene in a cell, after the molecule is introduced into the cell. For example, an siRNA molecule targets and binds to a complementary nucleotide sequence in a single stranded target RNA molecule. By convention, when an siRNA molecule is identified by a particular nucleotide sequence,

the sequence refers to the sense strand of the duplex molecule. One or more of the ribonucleotides comprising the molecule can be chemically modified by techniques known in the art. In addition to being modified at the level of one or more of its individual nucleotides, the backbone of the oligonucleotide can be modified. Additional modifications include the use of small molecules (e.g. sugar molecules), amino acids, peptides, cholesterol, and other large molecules for conjugation onto the siRNA molecule.

“Peptide Docking Vehicle” (PDoV) refers to a synthetic peptide of defined sequence that contains multiple conjugation sites to allow conjugation with one or more targeting ligands and with one or more oligonucleotides. It contains functional groups, such as a hydrophobic chain or a pH sensitive residue, which facilitate the release of the oligonucleotide payload entrapped inside of the endosome of a cell after delivery of the conjugated PDoV to the cell.

Compositions and methods using interfering RNA molecules having enhanced therapeutic benefit are provided. The compositions and methods allow targeted cell/tissue delivery of a therapeutic compound, such as an siRNA molecule, to a subject by linking a targeting ligand to the compound. The subject may be an animal or a human.

In some embodiments, the targeting ligand as described herein may be conjugated to an endosome releasing peptide through an orthogonal bioconjugation method. The targeting ligand may particularly be used to improve the delivery of RNAi molecules to a selected target, such as the liver. In other embodiments, the targeting ligand(s) permit targeted delivery of RNAi molecules into other tissues, for example, in the skin and brain.

The targeting ligands as described herein may include one or more targeting moieties and one or more linkers. The linkers are covalently conjugated with the siRNA and targeting ligands through click chemistry, thiol/maleimide chemistry, or other bioorthogonal chemistry. Linkers advantageously are hydrophilic and can be, for example, a water soluble flexible polyethylene glycol (PEG) which is sufficiently stable and limits the potential interaction between one or more targeting moiety(s). PEG has been validated to be safe and compatible for therapeutic purposes from clinical studies. In some embodiments, the linker can be poly(L-lactide)_n (where $n = 5-20$) of defined molecular weight, where the ester bond is enzymatically or hydrolytically labile.

The targeting ligand may include one or more targeting moieties, one or more groups with a linker reactive connection moiety. They are covalently conjugated with the siRNA and targeting ligands through click chemistry, thiol/maleimide chemistry, or other bioorthogonal chemistry. The linker reactive connection moiety may be, but is not limited to, a thiol-

maleimide linkage, a triazol linkage formed by reaction of an alkyne and an azide, and an amide formed from an amine-NHS ester linkage. Each of these linkages is suitable for covalently linking both the targeting ligands and the therapeutic compound.

In some embodiments, the targeting ligands disclosed here include one or more
5 targeting moieties, one or more linkers with reactive connection moiety. The linker contains a thiol moiety, or maleimide moiety, carboxylic acid, or amine, azido group, alkyne group, and the like.

In some embodiments, the targeting specific RNA compound disclosed herein can be directly conjugated to an endosome releasing docking peptide via the 3' or 5' terminal end of
10 the RNA. The targeting ligand (for example N-acetyl-galactosamine) also be conjugated with the same docking peptide in a compatible method.

In some embodiments, the targeting specific RNA compound disclosed herein can also be directly conjugated to a targeting ligand (for example N-acetyl-galactosamine), via, for example, the 3' or 5' terminal end of the RNA. In some embodiments, the RNA may
15 contain one or more modified nucleotides such as 3'-OMe, 3'-F, or 3'-MOE. In some embodiments, the RNA can be an RNAi agent, for example a double stranded RNAi agent. In some embodiments, the targeting ligands disclosed herein are linked to the 5' or 3' terminus of the sense strand of a double stranded RNAi agent or the 5' or 3' terminus of the antisense strand of a double stranded RNAi agent. The targeting ligands may alternatively be linked to
20 both 3'/3'', 3'/5' or 5'/5' terminal end of the sense and antisense strand of a double stranded RNAi agent.

The targeting ligands may be covalently bonded to the RNAi molecule via, for example, a phosphate, phosphorothioate, or phosphonate group at the 3' or 5' terminus of the sense strand of a double stranded RNAi agent. In some embodiments, the targeting specific
25 RNA compound disclosed herein is a TGF β 1 or an ApoB100, mRNA expression-inhibition specific compound.

The PDoV enhances escape of its macromolecular cargo into the cellular cytoplasm in a non-toxic manner. This allow effective delivery of, for example, RNAi therapeutics. An endosomal escape peptide (PDoV) is provided that enhances escape of macromolecular
30 cargo, such as an siRNA molecule, into the cytoplasm in a non-toxic manner. Various examples of the PDoV platform are shown in Figures 1-4. In the PDoV the endosomal escaping peptide acts both as the docking site linker for the RNA and the targeting ligands. Multiple RNA molecules can be conjugated with the same construct to achieve codelivery of siRNA molecules against different target mRNAs, thereby providing a synergistic benefit for

silencing a multi-disease related gene. The histidine and lysine rich polypeptide or linear histidine and lysine rich peptide has been shown to be an effective cell penetrating and endosomal release agent in the delivery of RNA. The peptide contains a histidine rich domain, where the imidazole rings of the histidine residues are protonated at a lower pH value (pH < ~6) and act inside the endosome as a proton sponge, which leads to lysis of the endosome lipid bilayers and release of the RNA. The conjugation sites on the PDoV are described in more detail below.

In the next few sections, each component will be discussed in detail including RNAi agent, targeting ligands, linkers between RNAi and peptide, linkers between ligand and peptide, and endosome releasing docking peptide.

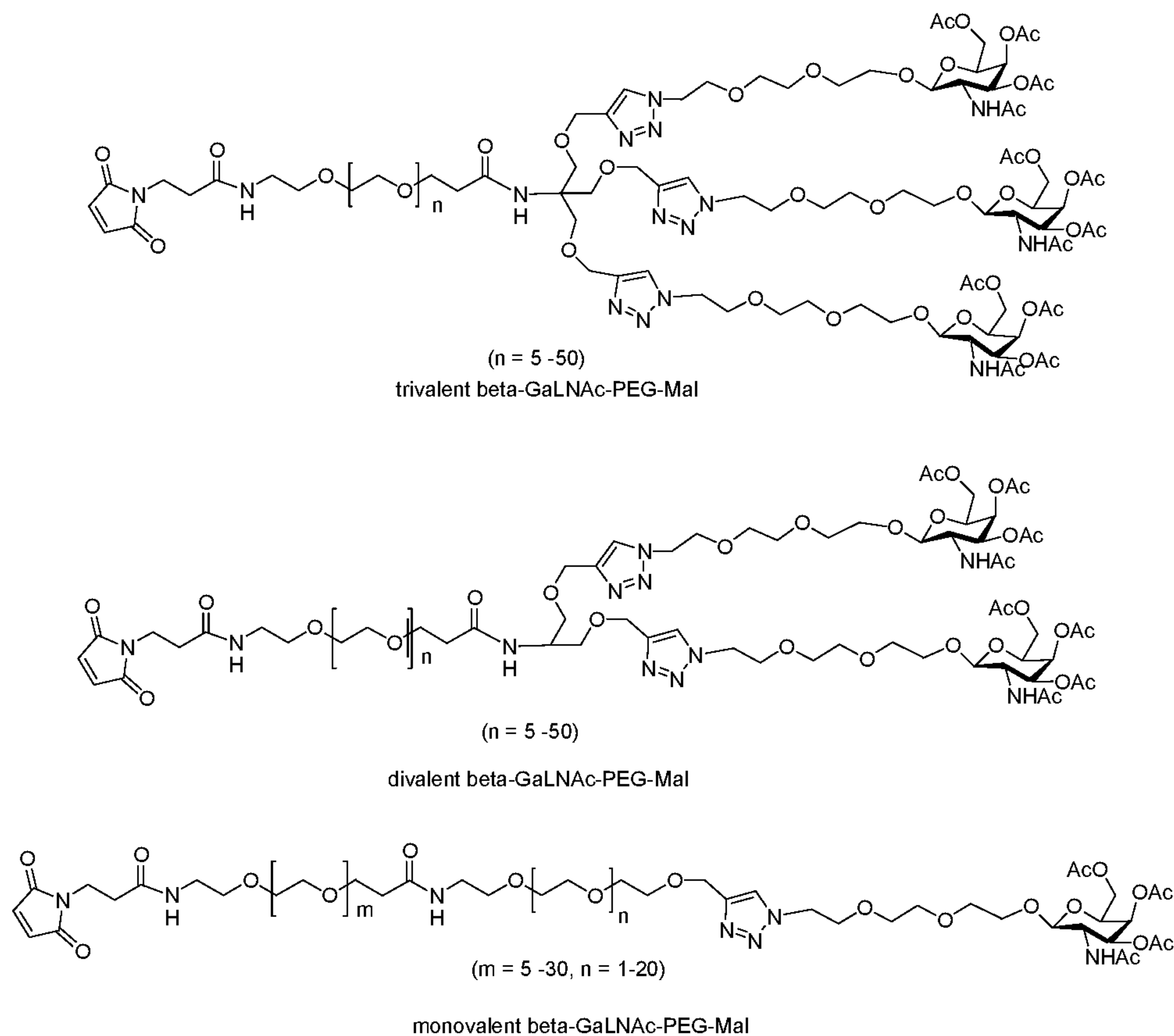
RNAi Agents

The RNAi molecules are double stranded compounds. For example, the double stranded siRNA can be anti-TGF β 1 or anti-ApoB100, and can be unmodified or chemically modified at the 2' position with, for example, 2'-OCH₃, 2'-F, or 2'-O-MOE, or at the 5' position with -P(O)₂S. Other chemical modifications are known in the art and can include, for example, pegylation or lipid functionalization to improve the overall stability and bioavailability of the RNAi.

In specific embodiments, the double stranded siRNA may be derived duplexes consisting of 24, 23, 22, 21, 20, 19, 18, 17 or 16 contiguous base pairs of any one or more of the duplexes in Figure 14.

Targeting Ligands

The targeting ligand moiety may be, for example, N-acetyl-galactosamine (GalNAc), galactose, galactosamine, N-formal-galactosamine, N-propionyl-galactosamine, N-butanoylgalactosamine, cRGD, GLP peptide or other small molecules. The targeting ligands are covalently coupled to the peptide by a covalent bond. The number ligands can be 1, 2, or 3. The targeting ligands disclosed here were has a structure represented by the following:



Linkers between RNAi and peptide

Linkage for the ligand conjugation Rs: Chemical group Rs may be one of various “click” like reactive moieties used to conjugate the oligonucleotide with the PDoV peptide vehicle. Rs can be amine, hydrazine, N-hydroxysuccinimide, azido, alkyne, carboxylic acid, thiol, or maleimide, or other chemical reactive moieties known in the art. Representative examples are shown in Figure 9.

The linker 2 in the conjugation Rs-linker2-siRNA is a chemical spacer disposed between the peptide and the conjugation site, which allow the conjugation site to be attached at the linker’s terminal site. The linker 2 can be an aliphatic chain or a polyethylene glycol chain, or other hydrophobic lipid or hydrophilic chain. The group 2 at the end site is the reactive site for the chemical conjugation with the siRNA end.

Linkers between Ligand and peptide

The targeting ligand and the RNAi moiety disclosed herein contains a linker-1, which directly connect the siRNA (3' or 5' end of the sense strand) and the bridge that contains the linker3- ligandslinks to the RNAi by the 3' or 5' terminal end the sense strand. The spacing of the linker-1 is a structure of selected from the linear polyethylene glycol, wherein n can be the number of ethylene glycol units in is 1 to 50, or poly(L-lactide) wherein the n can be

the number of repeating units of ethyl ester is between from 1 to 50 or average molecular weight from 100 to 3500. The conjugation site can be the maleimide/thiol or selected from the Group 2 list in Figure 10.

Endosome Releasing Docking Peptide

5 The Peptide Docking Vehicle (PDoV) advantageously has one ligand conjugation site together with multiple oligonucleotide sites. The PDoV has a peptide backbone with the general structure: $(H_nK_m)_oX_pY_q$ with multiple repeating units of histidine (H), lysine (K) and functional units X and Y (where X or Y is an amino acid, or an amino acid derivative selected from Linker 1 and the functional groups shown in Figure 10, and where: $n=1-10$;
10 $m=1-10$; $o=1-10$, $p=1-5$, and $q=1-5$. Note that Y in the context of a PDoV does not refer to the amino acid tyrosine – rather it defines a functional amino acid or linker. HK repeating units have been demonstrated to facilitate endosome release. The lysine residues or the functional unit(s) X may be used as docking sites for the conjugation of ligands and Y provides docking sites for the conjugation of oligonucleotide via a different covalent linkage.
15 Figure 3 shows a schematic of how the PDoV may be conjugated. For example, site ① is only able to react in the presence of ligand such as GalNAc or other targeting ligands. Site ③ can only conjugate with oligonucleotide and siRNA under selected conditions, as exemplified in Figure 10.

 Alternatively, the PDoV may have three ligand conjugation sites and multi
20 oligonucleotide sites (see Figure 4): a $(H_nK_m)_oX_pY_q$ peptide back bone has multi-repeating units of histidine (H), lysine (K) and functional units X and Y (amino acid, or functional linker), where the $n=1-10$, $m=1-10$, $o=1-10$, $p=1-5$, $q=1-5$. HK repeating units have been demonstrated to have good cell penetrating ability and to facilitate endosome release. The lysine or the various functional units X are adapted as the docking sites for the
25 conjugation of ligands, and Y is adapted the docking sites for the conjugation of oligonucleotides through different covalent linkages. For example, the site ① will only be able to react in the presence of a ligand such as GalNAc or other targeting ligands. The site ③ can only conjugate with oligonucleotides and siRNAs under specific conditions. See Figure 4.

30 In the structure design, the PDoV construct is an endosome releasing peptide inserted with multiple conjugation sites X and Y. Site X is used to conjugate the targeting ligand, and site Y is used to conjugate multiple oligonucleotide or nucleic acid. Some examples of the

constructs for the PDoV are shown in Figure 5, where: A represents peptide sequence K, R, H, HH, HHH, HHHH, HHK, HHHK or other short peptide; B represents peptide sequence K, R, H, HH, HHH, HHHH, HHK, HHHK, or other short peptide or other amino acid or combination; D represents oligonucleotide, siRNA, mRNA, or aptamer; RL represents ligand; and RS represents a linker to the oligonucleotide. In some embodiments, the peptide contains 5-15 amino acids.

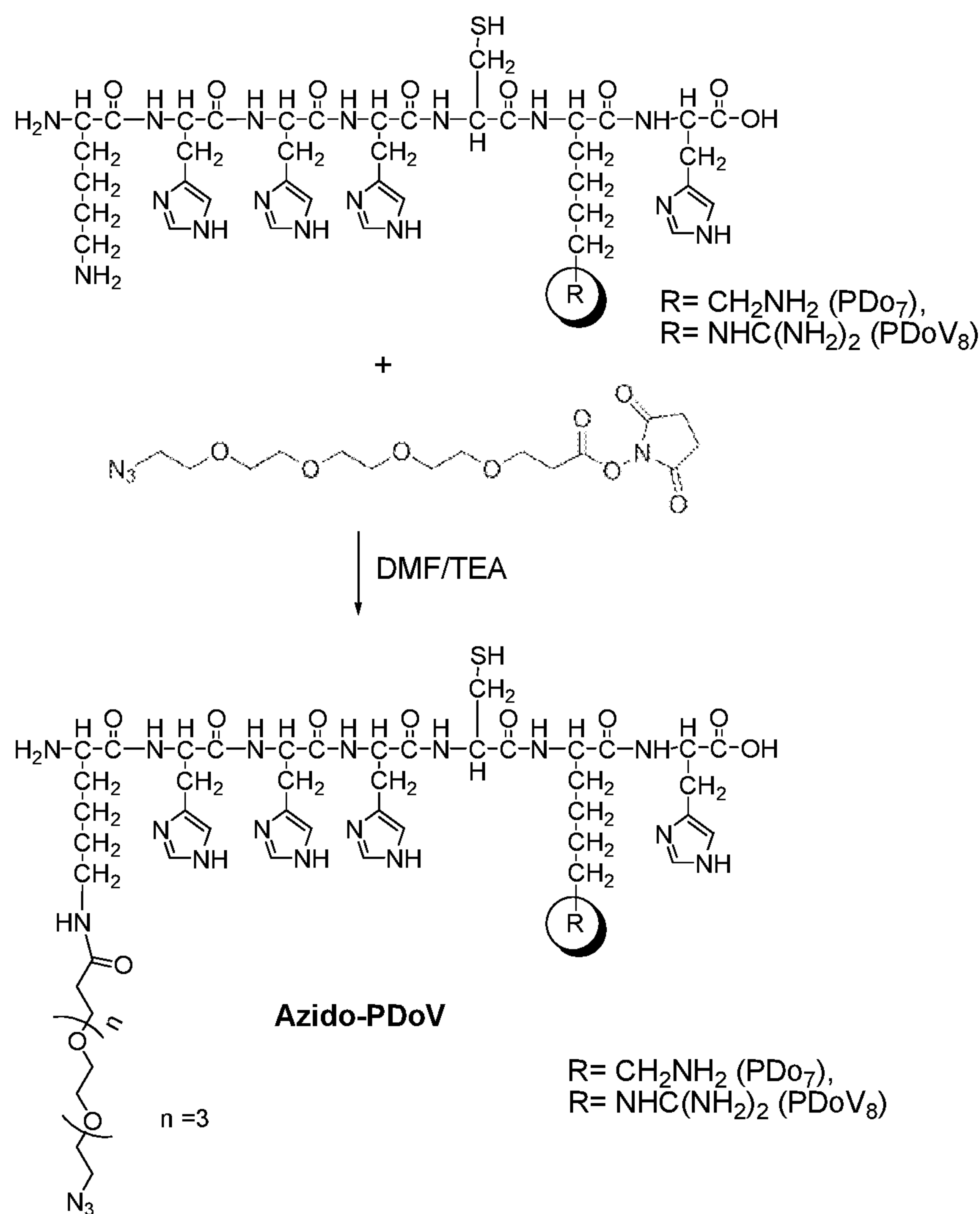
In some embodiments, the PDoV has a structure as shown in Figure 6.

EXAMPLES:

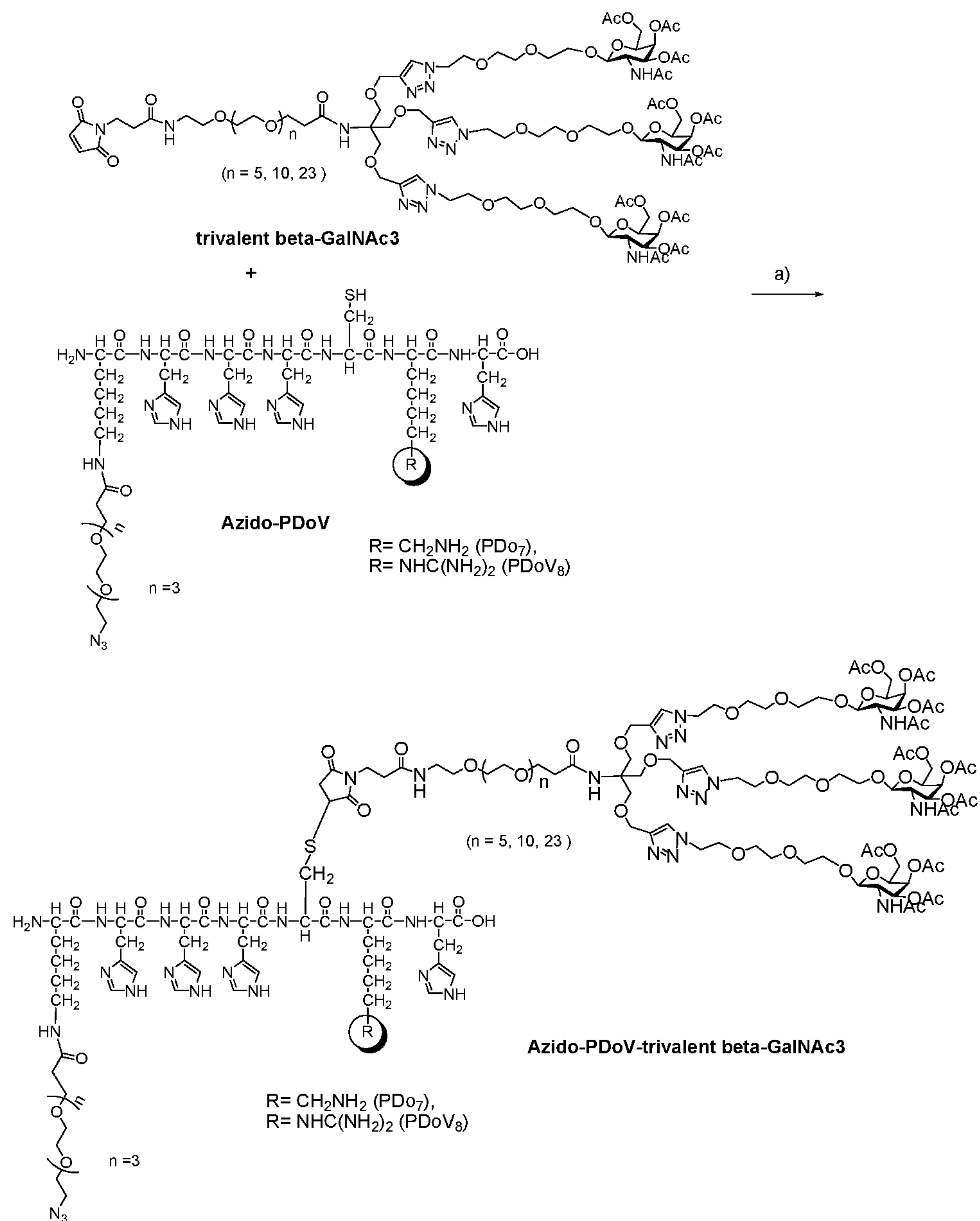
Example 1.

10 Schematic representative example of construction of the siRNA- PDoV-ligand compound.
One or two of the siRNA molecules can be conjugated with the PDoV peptide.

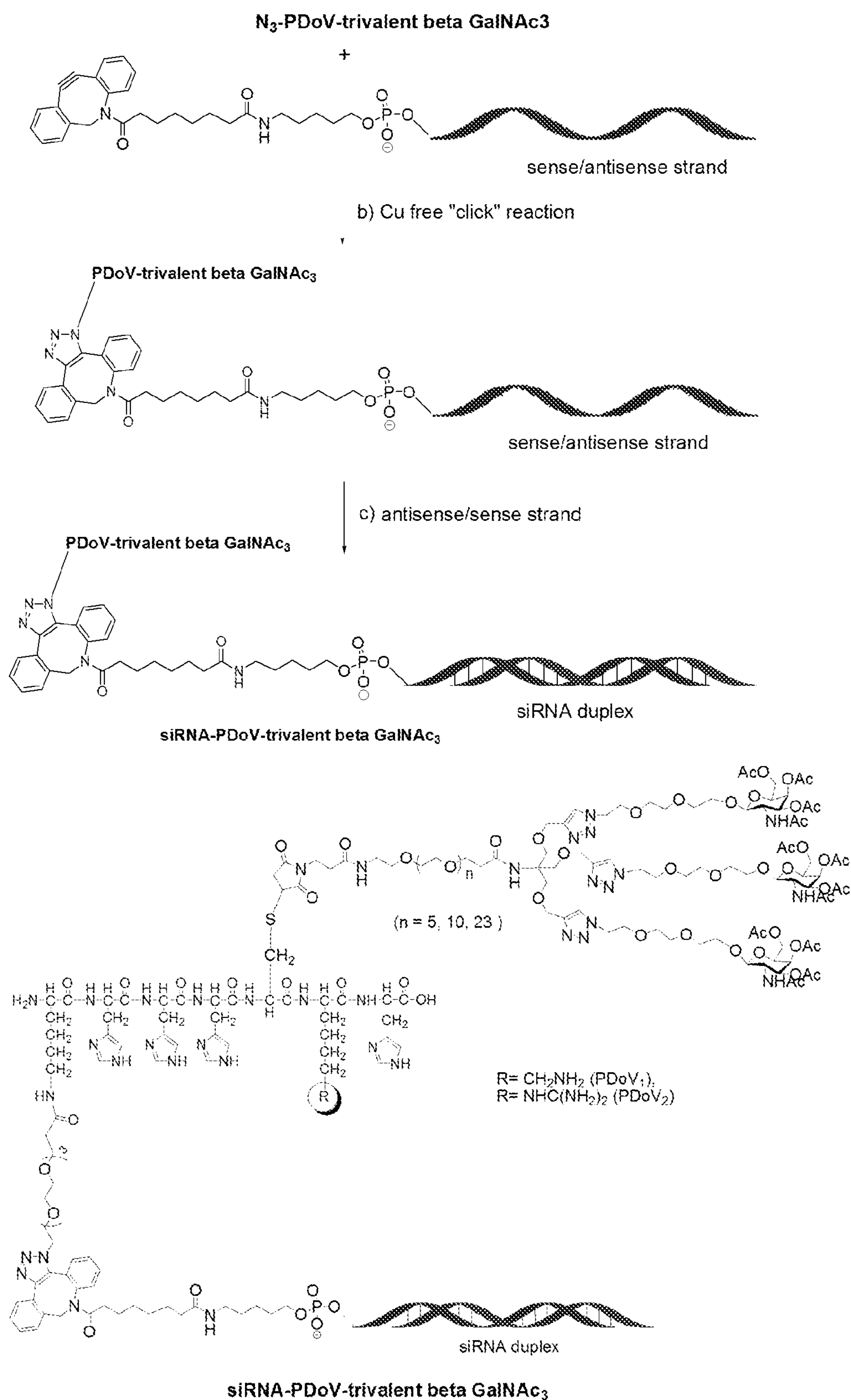
Step 1. Peptide azido modification:



Step 2. Conjugation of targeting ligand with the peptide



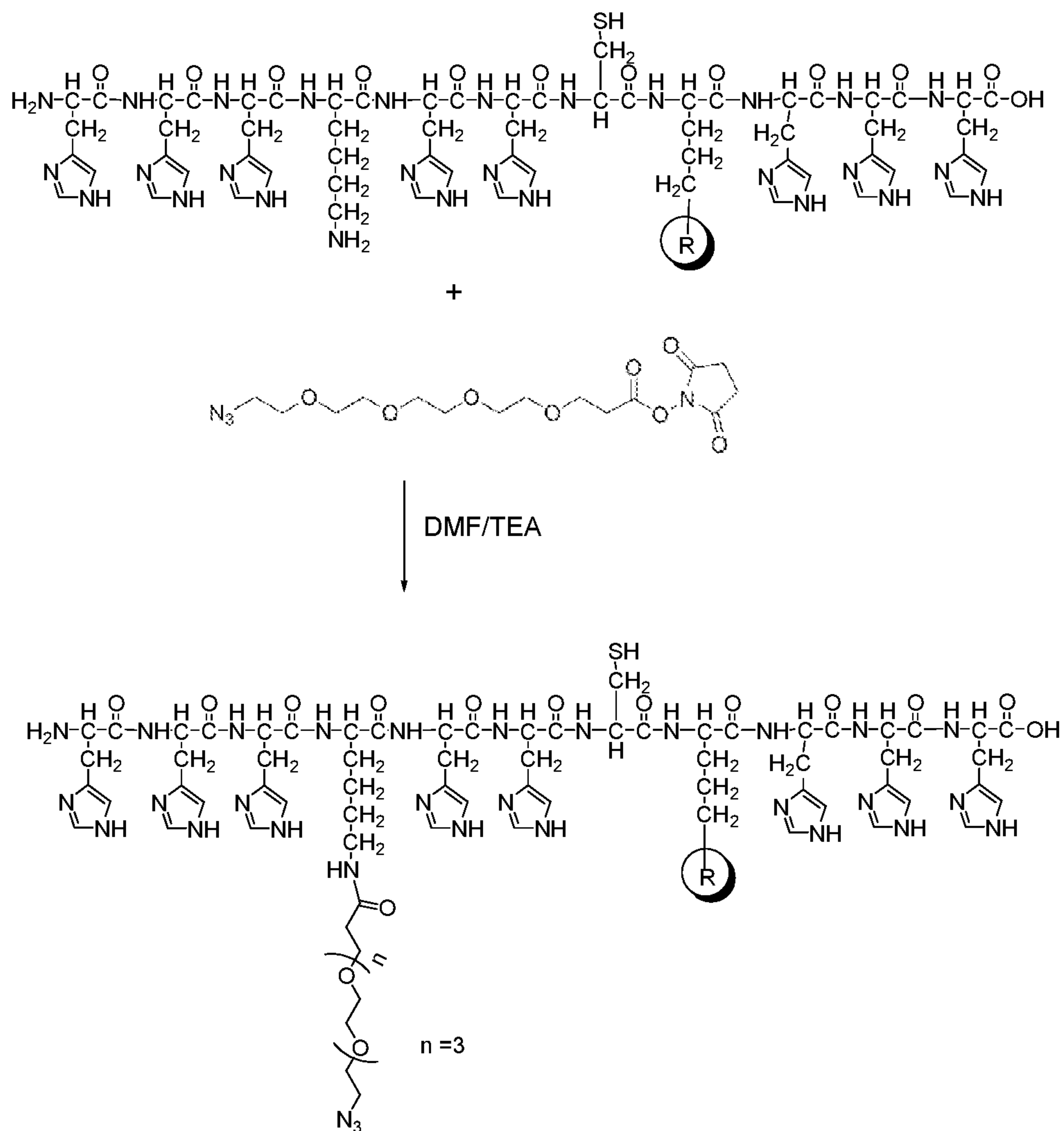
Step 3 and step 4. Oligonucleotide conjugation with the Azido-PDoV-GalNAc3



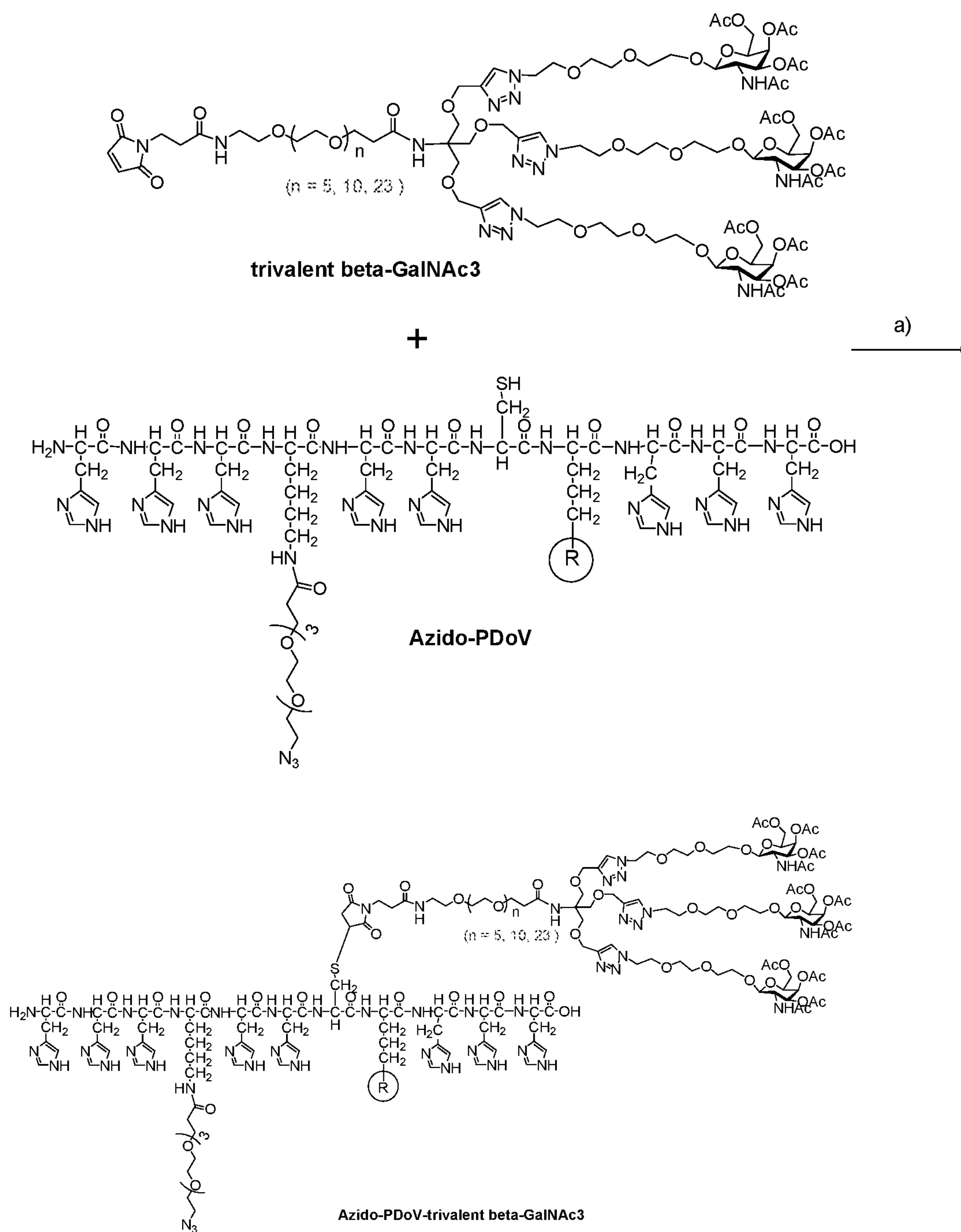
Example 2.

Schematic representative example of construction of the siRNA- PDoV-ligand compound.

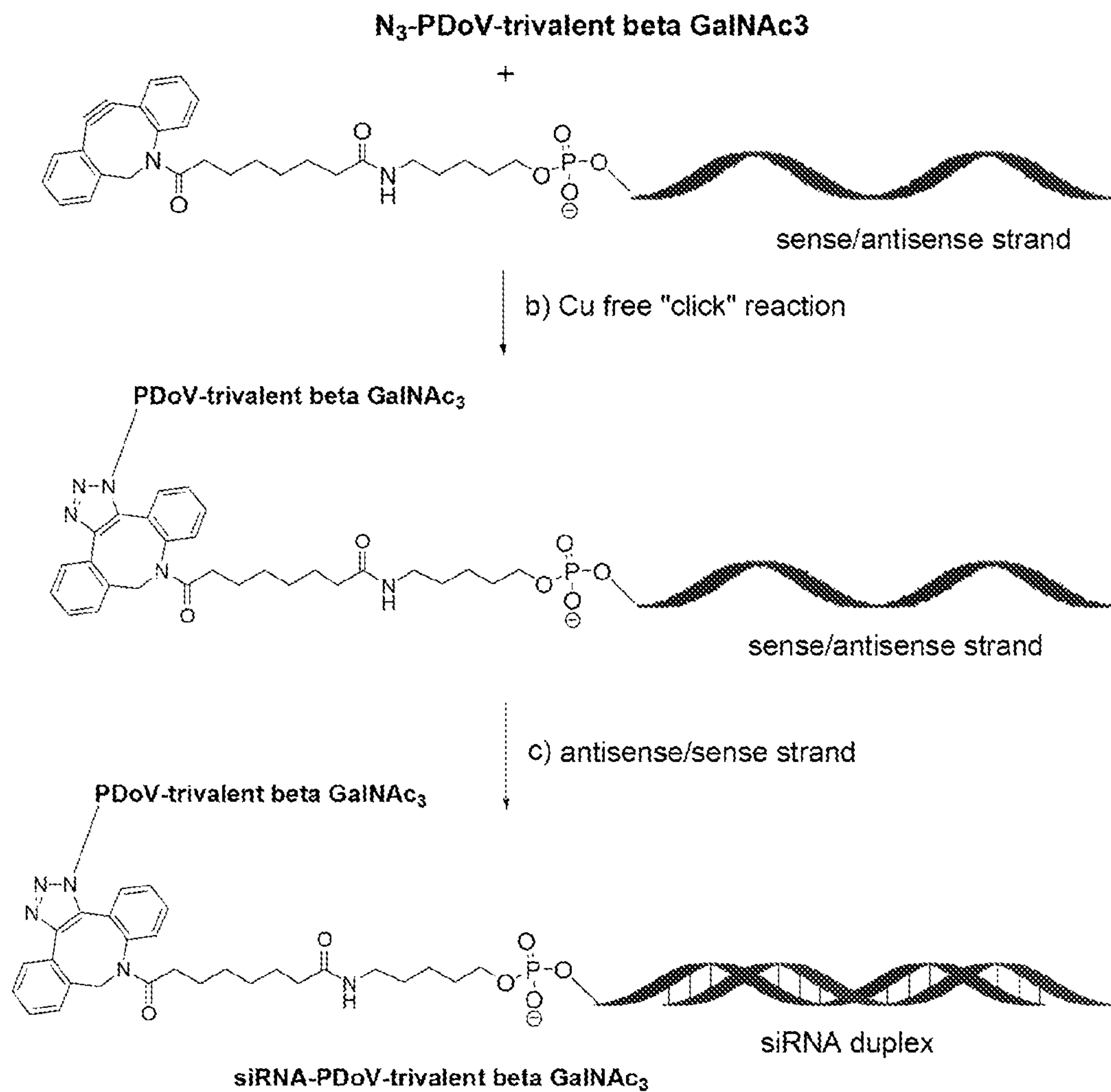
Step 1. Peptide azido modification.

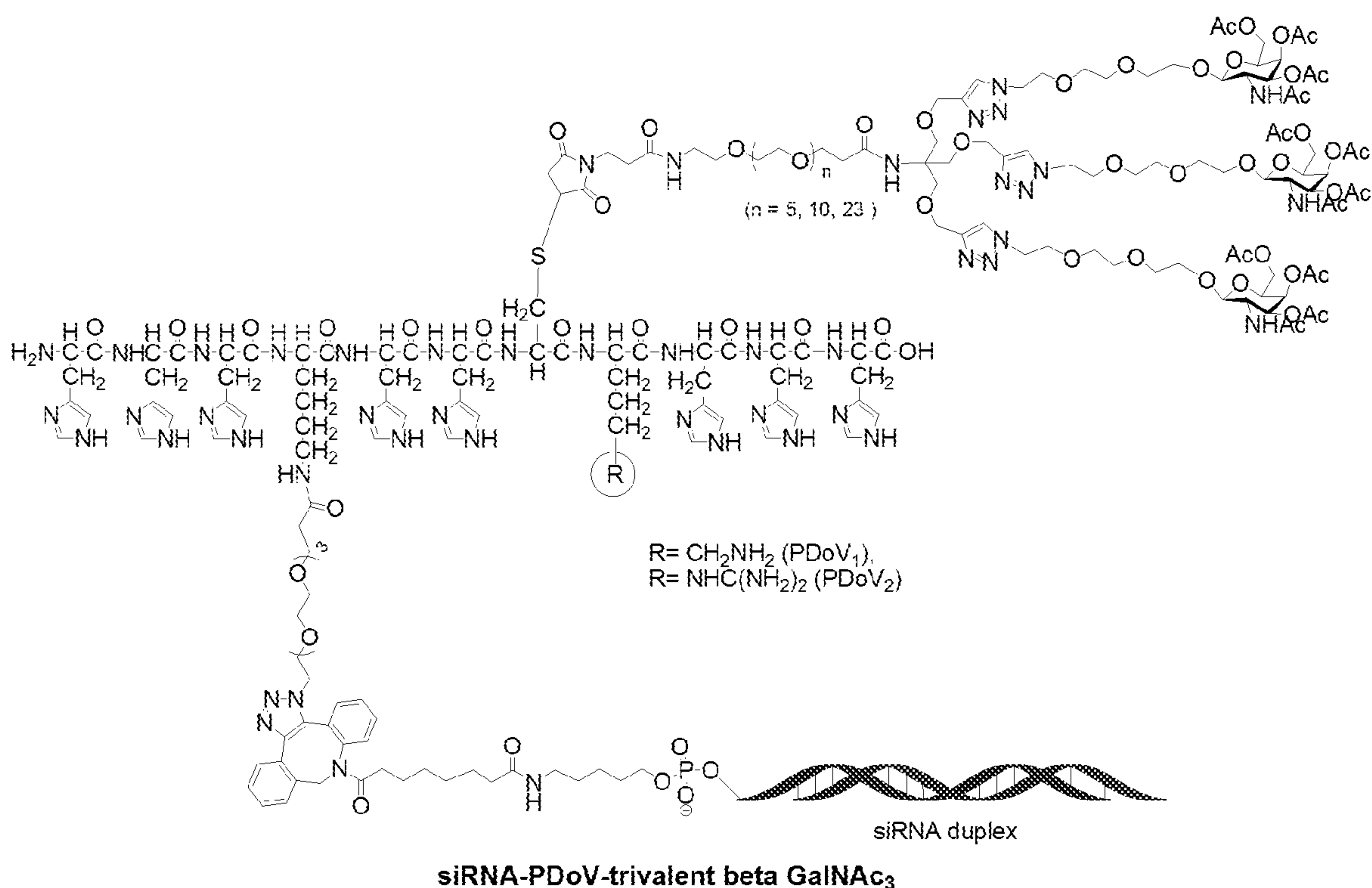


5 Step 2. Conjugation of targeting ligand with the peptide.



Step 3 and Step 4. Oligonucleotide conjugation with the Azido-PDoV-GalNAc3





Example 3.

Synthesis and characterization of Azido-PDoV1 (1):

5 Peptide **Azido-PDoV1** (sequence HHH{LYS(PEG4-N3)}HHCKHHH) was synthesized by the automated peptide synthesizer by contracted service and using standard amino acids and lysine-PEG4-N3 modifier in the sequence. The peptide was purified by C-18 reverse phase HPLC and characterized by mass spectrometry and ¹H NMR. The analytical data were all consistent with the expected structure.

10

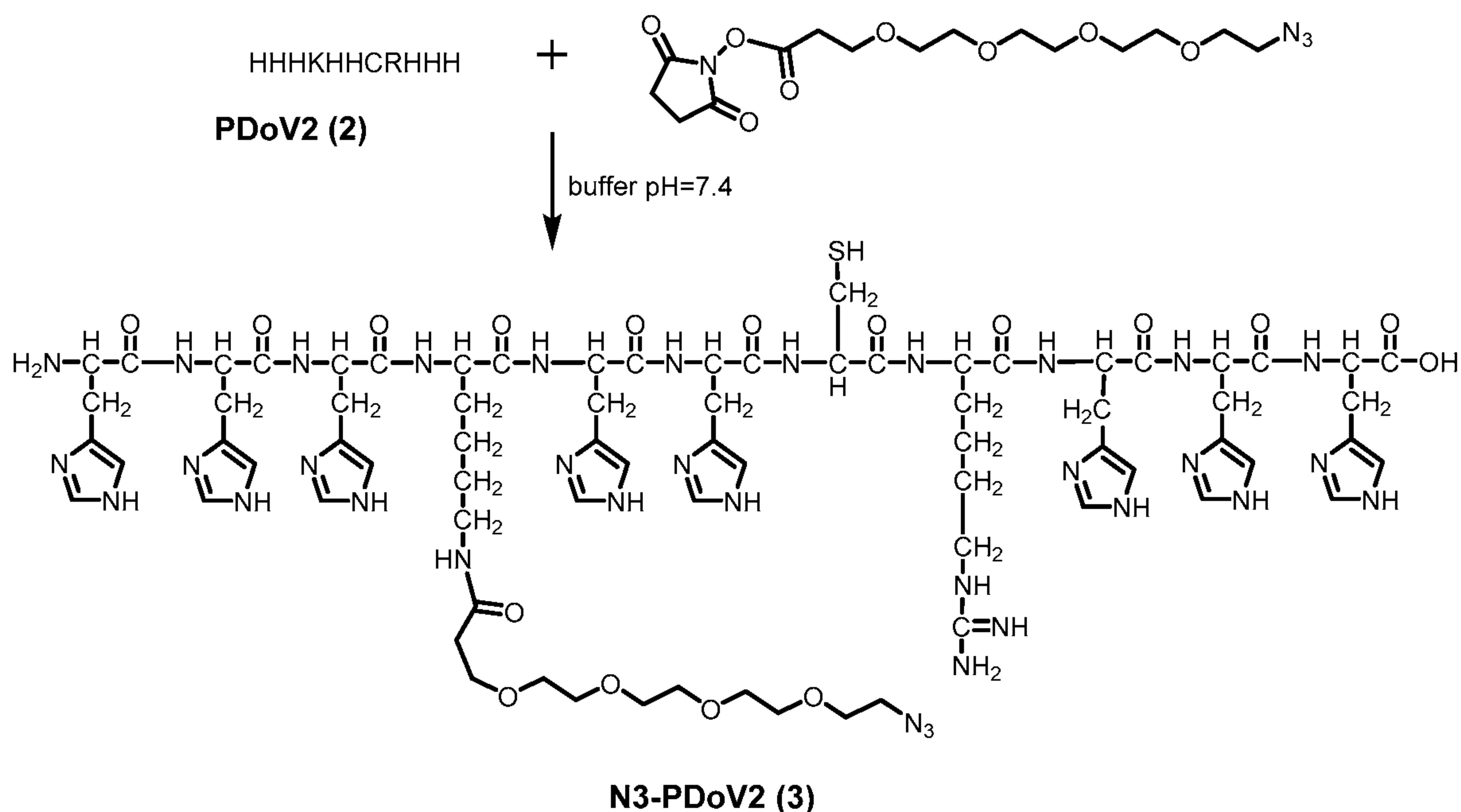
Example 4.

Synthesis of PDoV2 and Azido-PDoV2:

Synthesis of PDoV2 (2), sequence HHHKHHCRHHH. Peptide **PDoV2** (HHHKHHCRHHH) was synthesized by the automated peptide synthesizer by contracted service and using standard amino acids in the sequence. The peptide was purified by C-18 reverse phase HPLC and characterized by mass spectrometry. The analytical data were all consistent with the expected structure.

15

Synthesis of Azido-PDoV2 (3):



The azide linker was attached to the Peptide Docking Vehicle **2** (PDoV2) via amide bond formation between the ester activated carboxylic acid of the azide linker and the primary amine of the Lysine side chain of PDoV2 (**2**) to form compound **3**. PDoV2 peptide

5 HHHKHHCRHHH (42 mg, 0.0280 mmol) was suspended in 1.0 mL DMF. Triethyl amine (39 μL , 10 eq) was added and the mixture was stirred at room temperature for 20 minutes. A solution of Azido-Peg4-NHS ester (54 mg, 0.140 mmol, 5eq) in 20 μL of DMF was added to the reaction mixture. The reaction mixture slowly turned into a clear solution over 30 minutes and was stirred further at room temperature for 16 hours. The TLC profile of the reaction

10 mixture was monitored by HPLC profile of full complete conversion of PDoV2 (Fig. 1).

The reaction mixture was quenched with water (200 μL), concentrated using a rotary evaporator and the crude material was purified through HPLC on semi-prep RP-C18 column using an increasing gradient of 10-90% of Buffer B (0.1% TFA in Acetonitrile). Azido-PDoV2 (**2**) was isolated as the major product with a retention time between 10.5 and 11.5

15 minutes. Sample fractions were lyophilized resulting in a clear residual oil of compound **2** (44 mg, 88 % yield). Its proton and MS analysis were as follows: ^1H NMR (400 MHz, D_2O , fig. 2) δ 8.74 (brd d, 8H) and δ 7.35 (brd d, 8H) are consistent with aromatic hydrogens associated with 8 histidine tetrazoles in the peptide above δ 6.00 ppm. Methine hydrogens at the alpha carbon of all the 11 amino acids at δ 4.75-4.30 (brd t, 11H), ethylene hydrogens associated with polyethylene group at δ 3.90-3.75 (m, 100H); δ 3.6-2.75 (m, 53H) and δ 1.8-

20 1.3 (m, 12H) ethylene hydrogens associated with the side chain protons of Lysine, arginine and cysteine. In total, 104 hydrogens are observed in the non aromatic regions and are in

excess by 41 hydrogens. These extra hydrogens are the equivalent of 2 Azido-peg4 groups. The NMR data is consistent with ESI-MS data: expected m/z 1775.9 Da and the observed m/z 2323.5 Da (fig. 3). The observed mass to charge ratio is 547.6 Da units more than the expected value. The extra mass units are consistent with mass of two Azide-linkers.

5

Example 5.

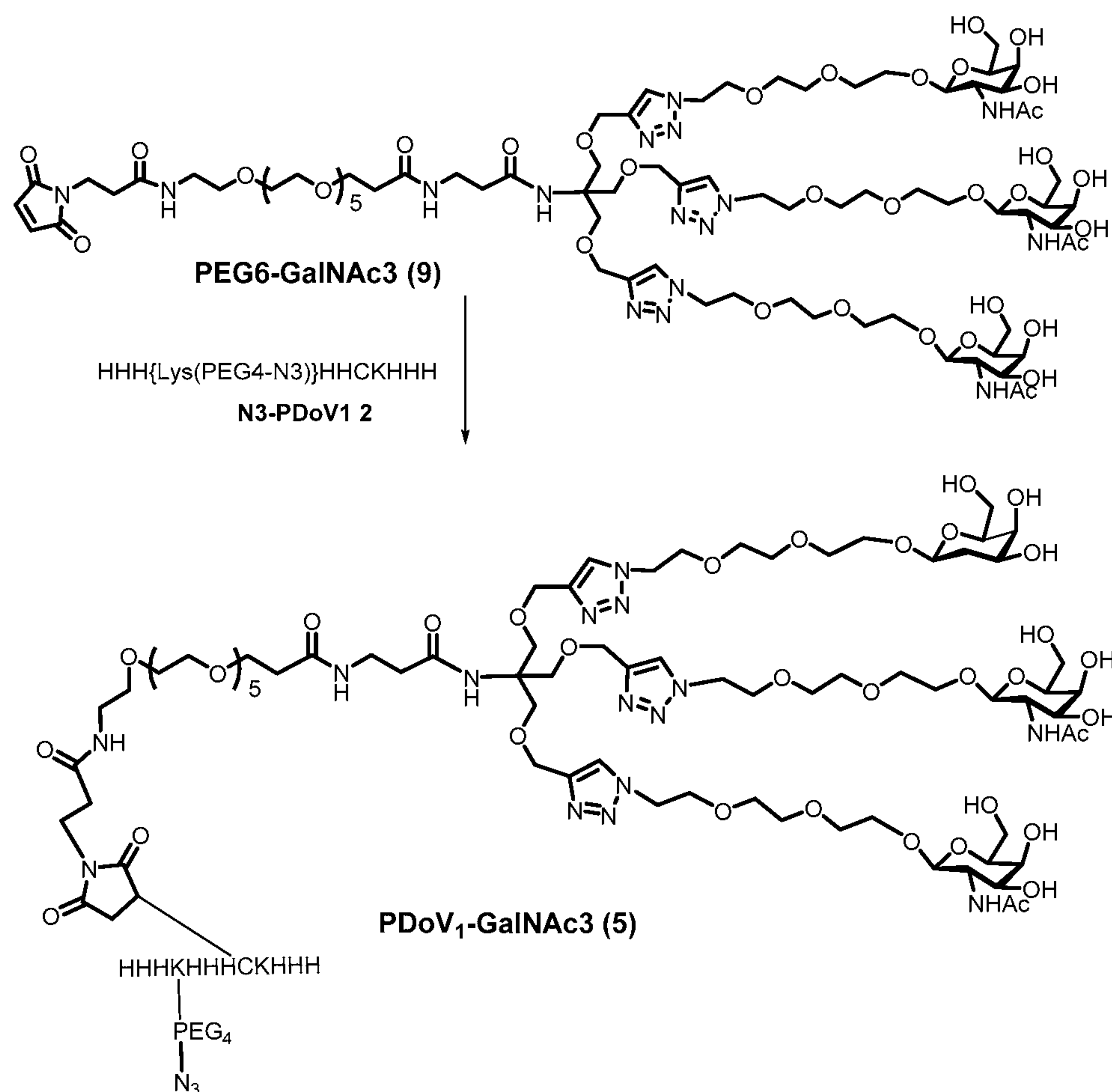
Synthesis and characterization of Azido-PDoV3 peptide (4).

Peptide **Azido-PDoV3** ({LYS(PEG4-N3)}HHHCHH) was synthesized using solid-phase automated synthesis using standard amino acids plus lysine-PEG4-N3 modifier in the sequence. The peptide was purified by C-18 reverse phase HPLC and characterized by ^1H NMR and mass spectrometry. The analytical data were all consistent with the expected structure.

10

Example 6.

Synthesis and characterization of PDoV1-GalNAc3 (5).



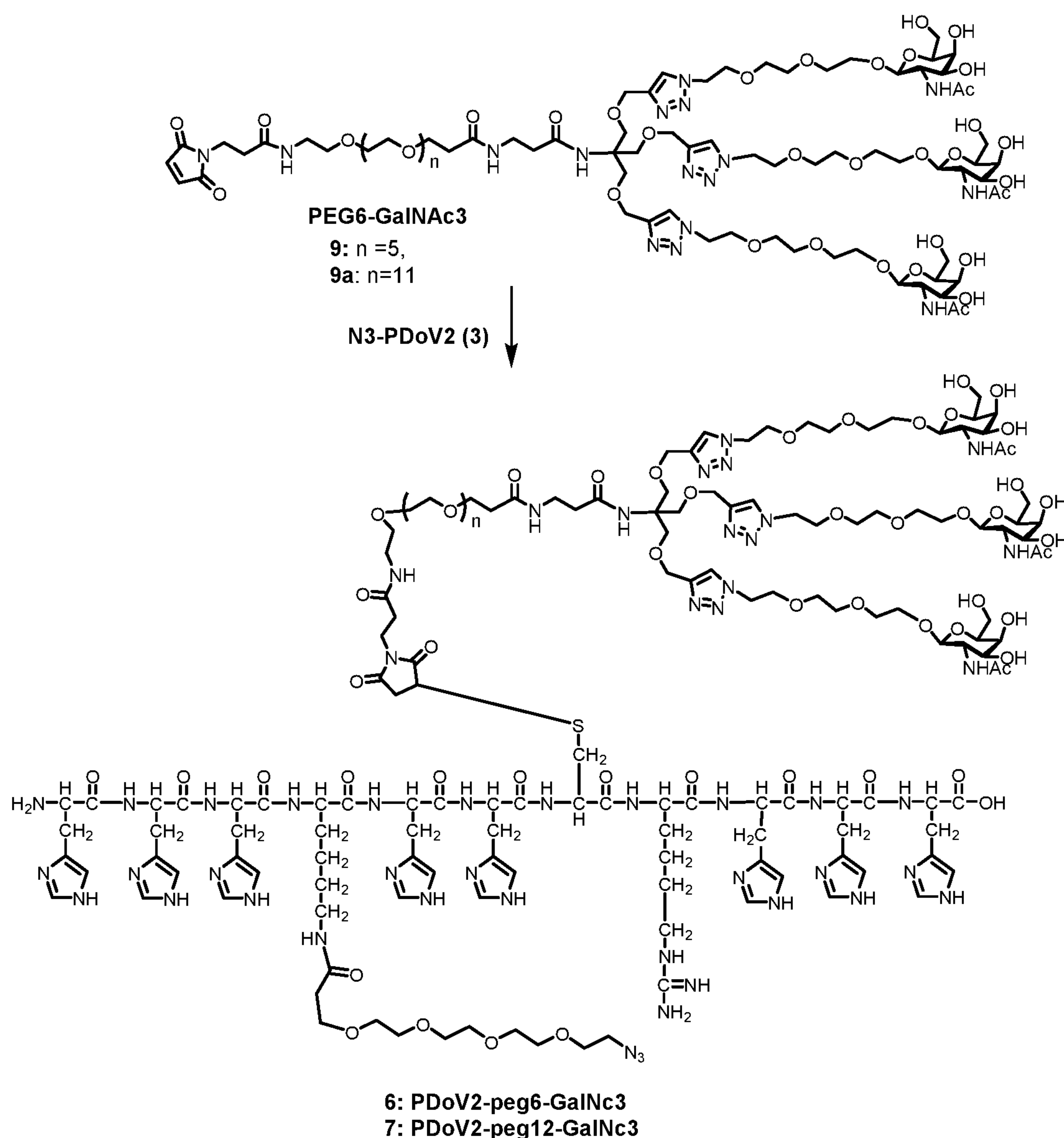
15

Scheme 1: Synthesis of **PDoV1-GalNAc3 (5)**.

PEG6-GalNAc3 (9) (3.0mg, 1.56 μ mol) in dry DMF (400 μ L) was added to the solution of **N3-PDoV1 2** (3.54 mg, 2.03 μ mol) in phosphate buffer (1 mL, pH=7.4). The resulting mixture was stirred at 25 °C under nitrogen overnight. After the solvent was removed under reduced pressure, the sample was desalted and purified by PD-10 column to provide the pure product **PDoV1-GalNAc3 5** (5.1 mg, white solid, yield 90%). The product was analyzed by HPLC using a reverse phase C18 column, gradient elution by solvent 0.1% TFA water and 0.1% acetonitrile. Retention time R_t =4.877min, purity > 90%. Mass spectrum analysis (ESI, positive): Calc. for $C_{154}H_{240}N_{48}O_{55}S$ 3673.7 found 3674.8.

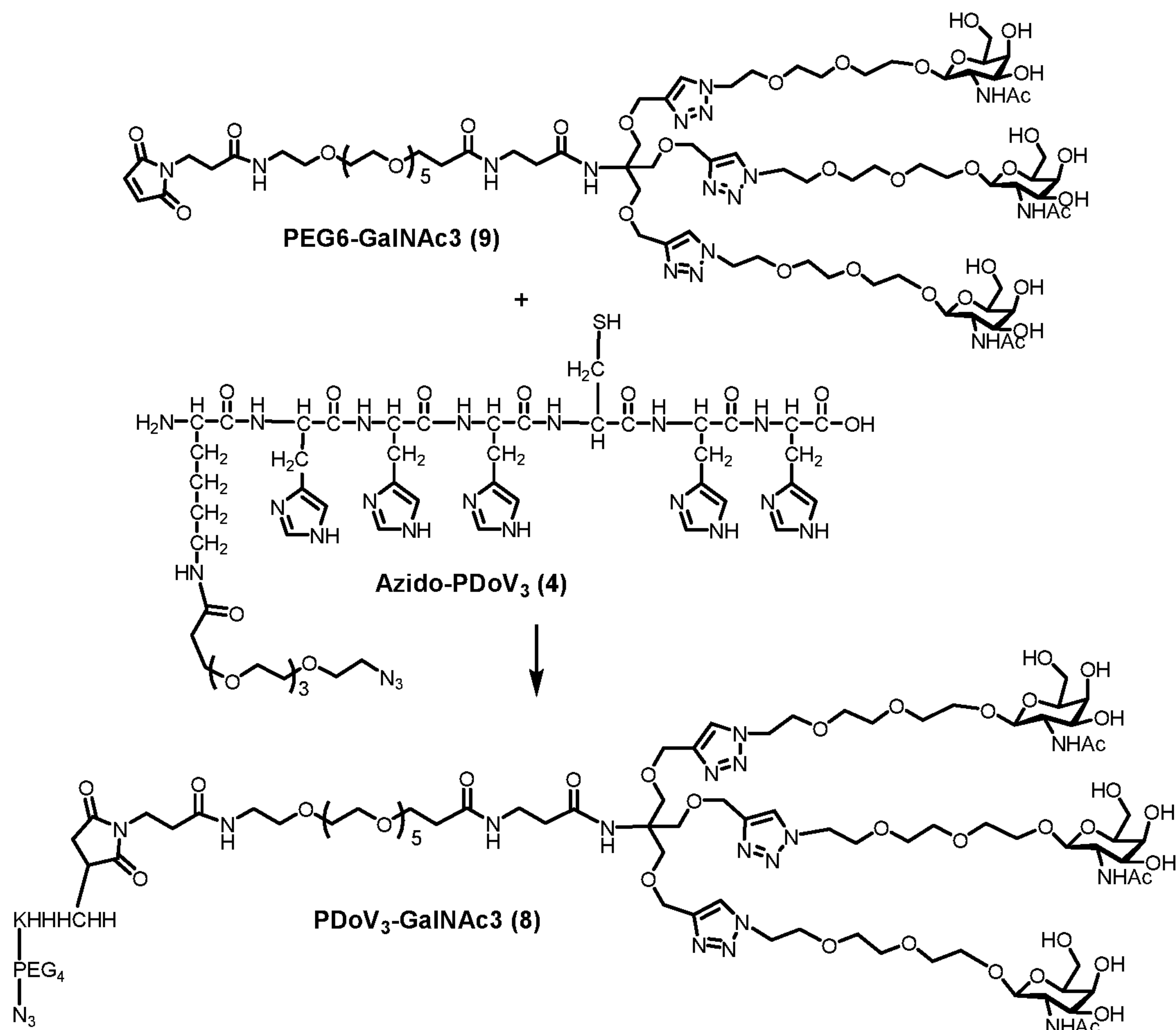
10 Example 7.

Synthesis and characterization of PDoV2-GalNAc3 6 and 7.

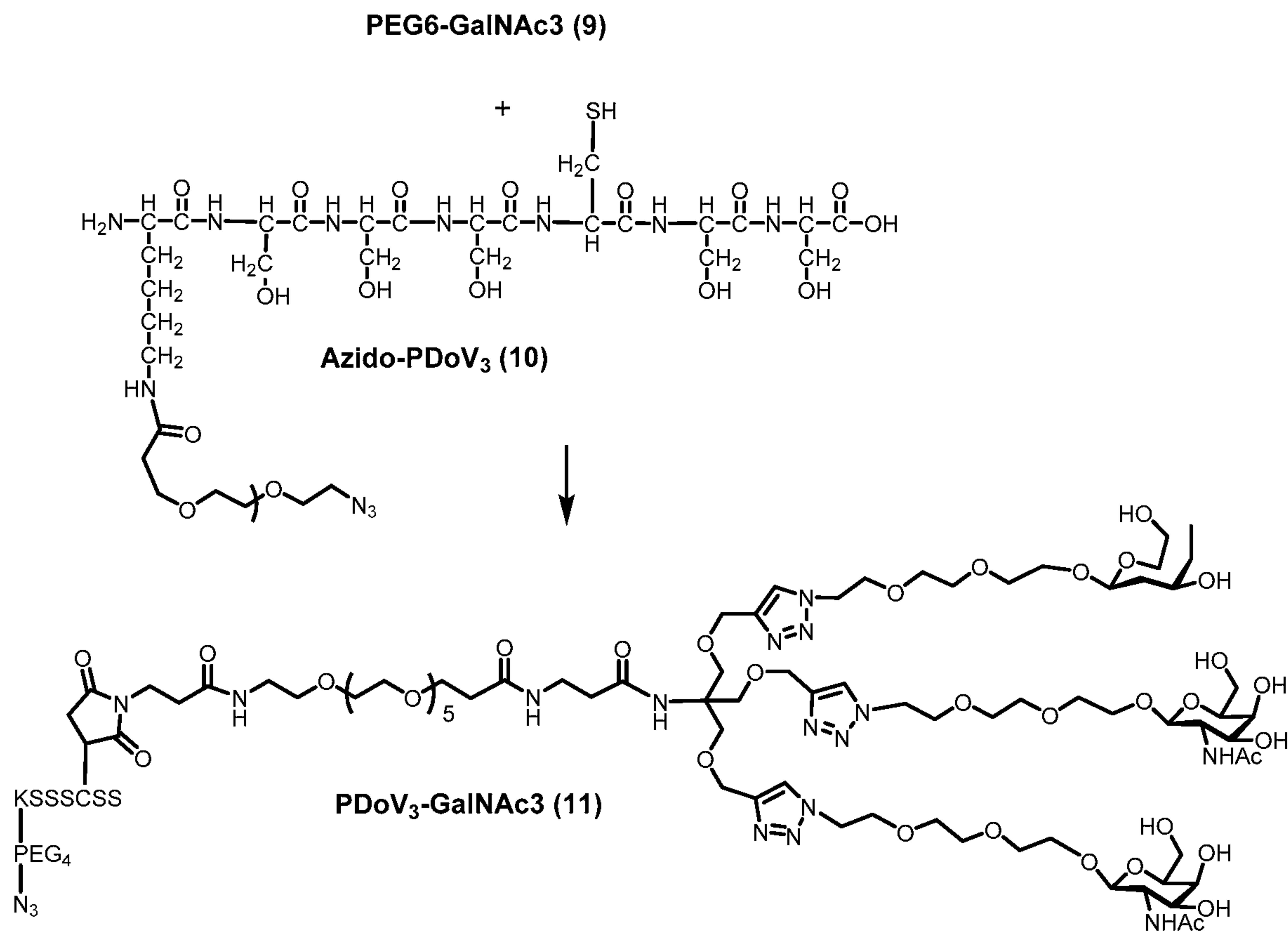


Scheme 2: Preparation of PDoV2-linker-GalNAc3.

- Preparation of PDoV2-Peg6-GalNAc3 (compound 6):** The nucleophile, Compound **2** (49.8 mg, 0.0243 mmol) was dissolved in 1.0 mL of degassed PBS buffer at pH 7.4. Trivalent GalNAc-ligand (**9**) (30.8 mg, 0.0160 mmol) was dissolved and delivered in 400uL of dry DMF. The reaction mixture was again degassed under dry argon and allowed to stir at room temperature overnight. The reaction mixture was quenched with water (100 μ L) and desalted through 1.0 μ mol Sephadex Nap column following the Glen Research recommended protocols. The eluent was lyophilized, and the crude material was eluted on HPLC through a semi-prep C18 reverse phase column with increasing gradient of 10-90% of Buffer B (0.1% TFA in Acetonitrile and water (fig. 7). The product had a retention time of 4.0 mins and was isolated as an oil (39 mg, 60 % yield). The mass spec of the modified oligos confirmed that synthesis of the PDoV2-peg6-GalNAc3 construct was successful.
- Preparation of PDoV2-Peg12-GalNAc3 (compound 7):** Compound **7** azido-PDoV2 (4.9 mg, 2.74 μ mol) was dissolved in 1.0 mL of degassed PBS buffer at pH 7.4. GalNAc-ligand (3.0 mg, 1.37 μ mol) was dissolved and delivered in 400uL of dry DMF. The reaction mixture was again degassed under dry nitrogen and allowed to stir at room temperature overnight. The reaction mixture was quenched with water (100 μ L) and desalted through 1.0 μ mol Sephadex Nap column following the Glen Research recommended protocols. The eluent was lyophilized, and the crude material was eluted on HPLC through a semi-prep C18 reverse phase column with increasing gradient of 10-90% of Buffer B (0.1% TFA in Acetonitrile and water (fig.4). The product was exclusively formed as a major product with a retention time at 4.2 minute and was isolated as a white powder (4.4 mg, 81 % yield). Proton NMR and MS analysis: ¹H NMR (400 MHz, D₂O,) δ 7.94(s, 3H, triazoles), δ 8.57- δ 7.19 (m, 18H, 8 histidine aromatic), δ 4.75-4.30 (m, 14H, amino acids), δ 4.5 (d, 3H, galactose), δ 2.05 (s, 9H) The NMR data is consistent with ESI-MS data. ESI-MS (positive mode, m/z) Calc. 3968.2 for C₁₆₈H₂₆₈N₅₀O₆₁S, observed 3968.2.

Example 8.**Synthesis of PDoV3-GalNAc3 (8).**Scheme 3: Synthesis of **PDoV3-GalNAc3 (8)**.

- 5 **Synthesis of PDoV3-GalNAc3 (compound 8):** Azido-PDoV3 compound **4** (47.0 mg, 38.9 μmol) in DMF (1.5 mL) was added to the mixture of trivalent GalNAc (**9**) (50.0 mg, 25.9 μmol) in phosphate buffer (4 mL, pH 7.4) under nitrogen at 25 °C. The resultant reaction mixture was stirred at 25 °C for 12 hours. The reaction was monitored by HPLC until GalNAc **9** was fully consumed. The solvent was removed by lyophilization and the crude
- 10 material was purified by gel permeation column chromatograph PD-10 column to provide the pure product **PDoV3-GalNAc3** compound **8** (70 mg, yield 86.4%). The HPLC was performed on reverse phase C-18 column by gradient elution of solvent 0.1% TFA in water and 0.1% TFA in acetonitrile R_t = 5.038 min. MS (ESI, positive mode) Exact Mass: 3134.45 for Formula: C₁₃₀H₂₀₇N₃₇O₅₁S. Found: 3136.35. The analytical data were all consistent with
- 15 the expected structure.

Example 9.Synthesis of Control3-GalNAc3 (11).

5

Scheme 4: Synthesis of Control3-GalNAc3 (11).

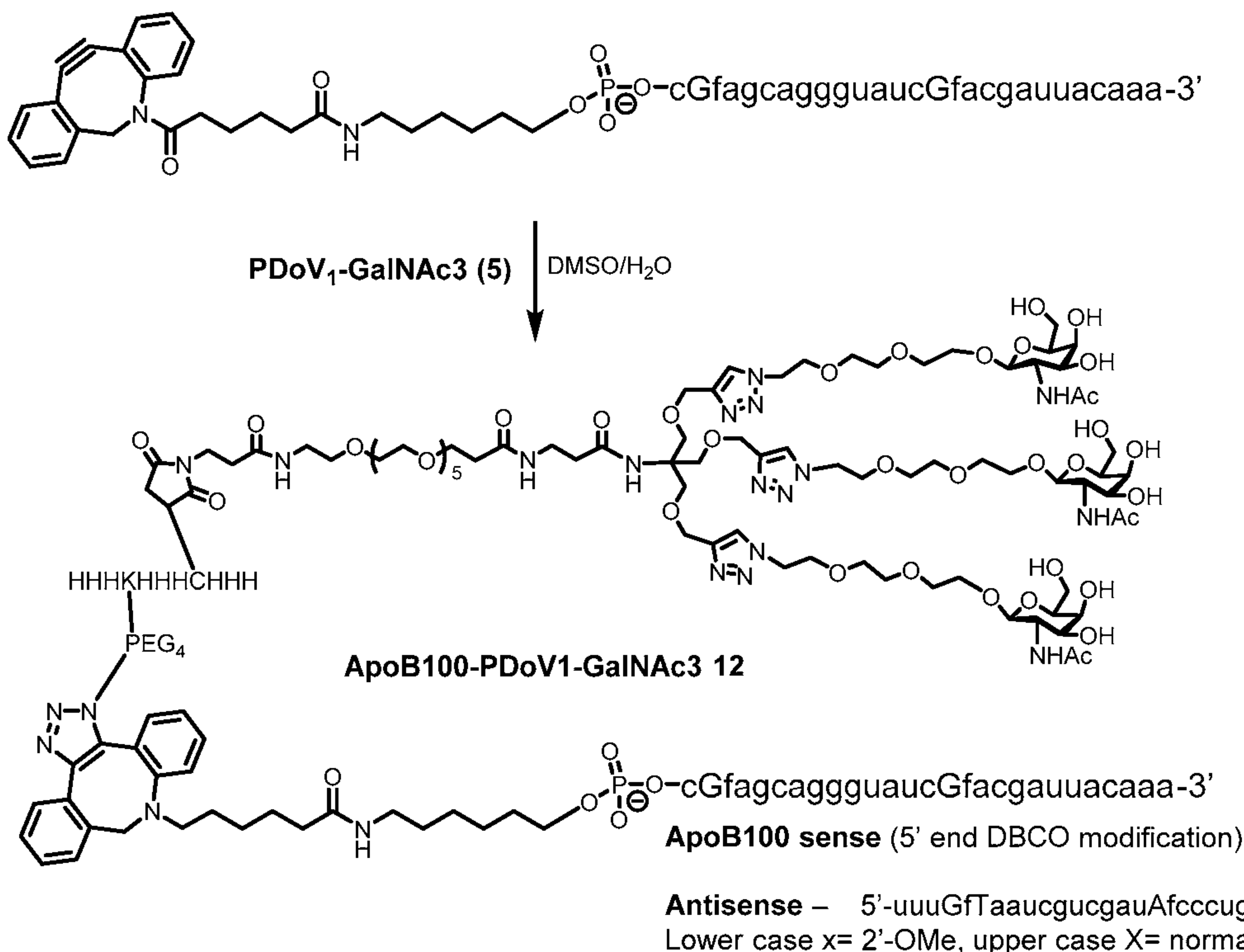
Preparation of PDoV3-control3-GalNAc3:

- 10 Compound azido-control3 peptide 11 (sequence {LYS(PEG4-N3)}SSSCSS) (2.6 mg, 2.59 μ mol) was dissolved in 1.0 mL of degassed PBS buffer at pH 7.4. GalNAc-ligand (5.0 mg, 2.59 μ mol) was dissolved and delivered in 500 μ L of dry DMF. The reaction mixture was again degassed under dry argon and allowed to stir at room temperature overnight. The reaction mixture was quenched with water (100 μ L) and desalted through 1.0 μ mol Sephadex
- 15 Nap column. Several eluent fractions were collected and lyophilized to afford the desired compound Control3-GalNAc3 10. This compound was analyzed using analytical HPLC C18 RP column with increasing gradient of 10-90% of Buffer B (0.1% TFA in Acetonitrile and water. The product had a retention time of 3.80 min and was isolated as a clear oil (4.9

mg, 67 % yield). The mass spectrum of the modified oligonucleotides confirmed the structure of the PDoV3-Control3-peg6-GalNAc construct.

Example 10.

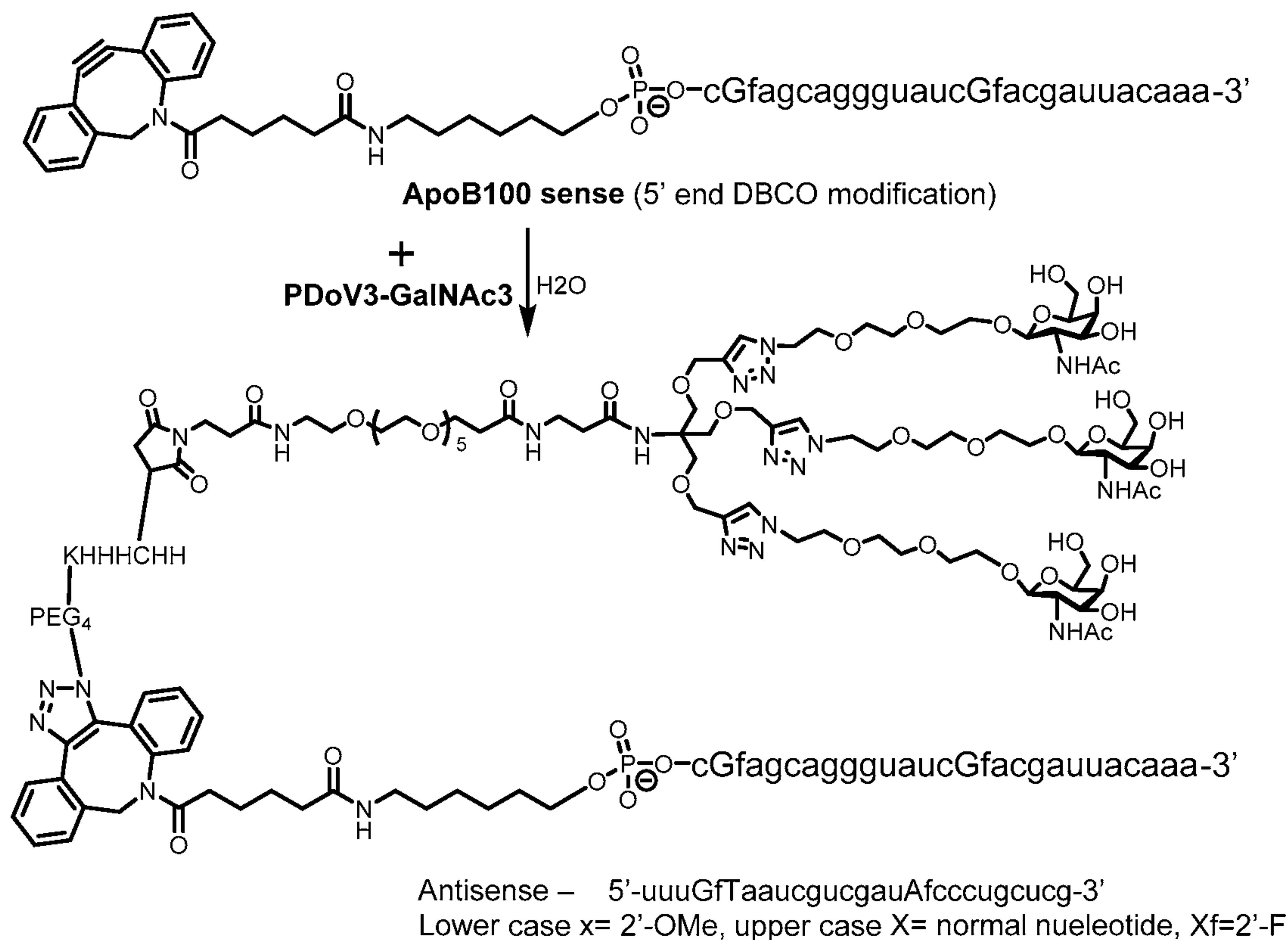
Synthesis and characterization of ApoB100-PDoV1-GalNAc3 (12).



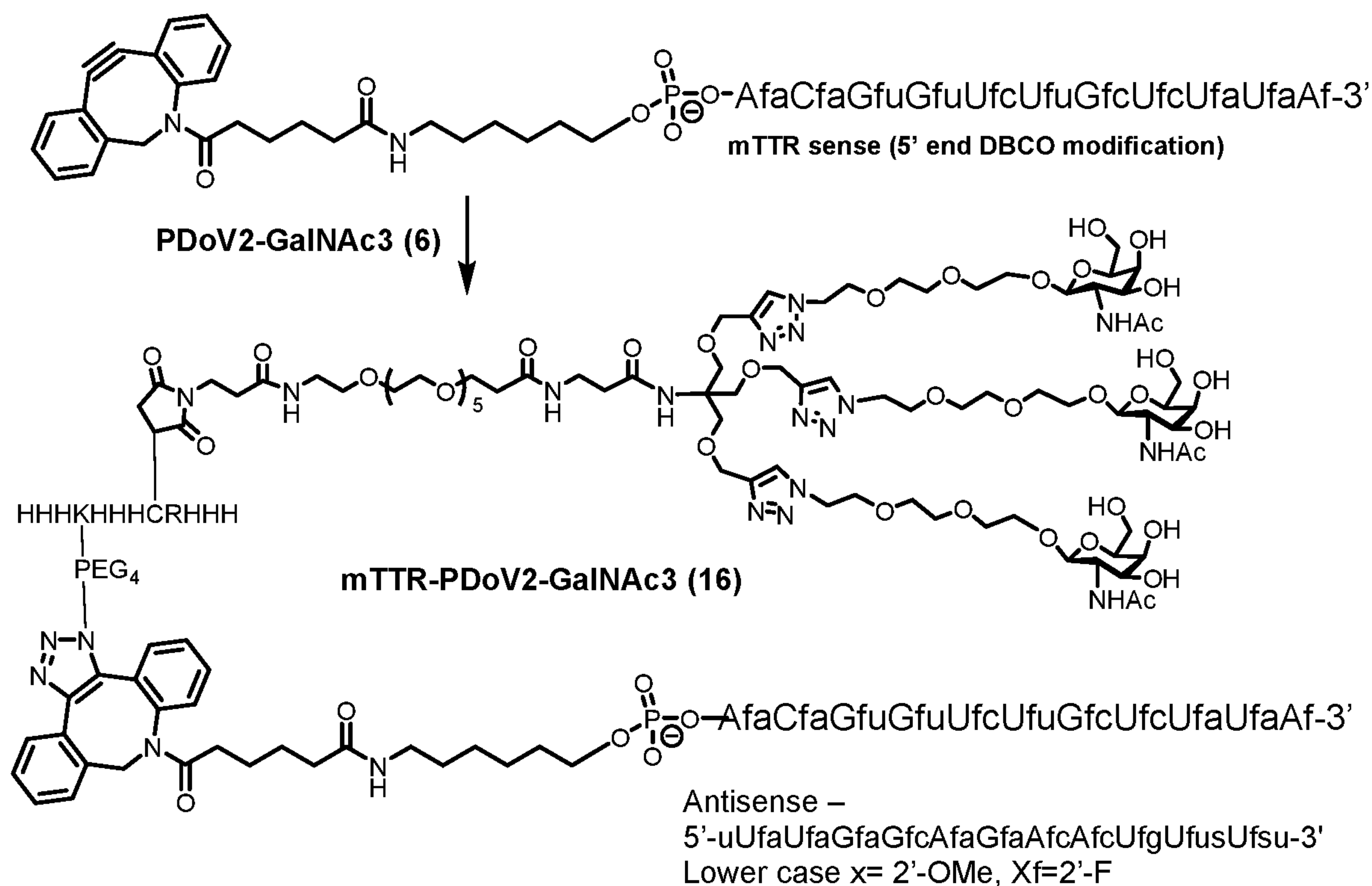
Scheme 5. Synthesis of ApoB100-PDoV1-GalNAc3 (12).

PDoV1-GalNAc3 5 (381 μ g, 0.104 μ mol) in DMF (95 μ L) was added to a solution of ApoB100-sense-5'-DBCO (500 μ g, 0.069 μ mol) in RNase free water (250 μ L). The resultant mixture was stirred at 25°C for 2 hours. After solvent was removed under reduced pressure,

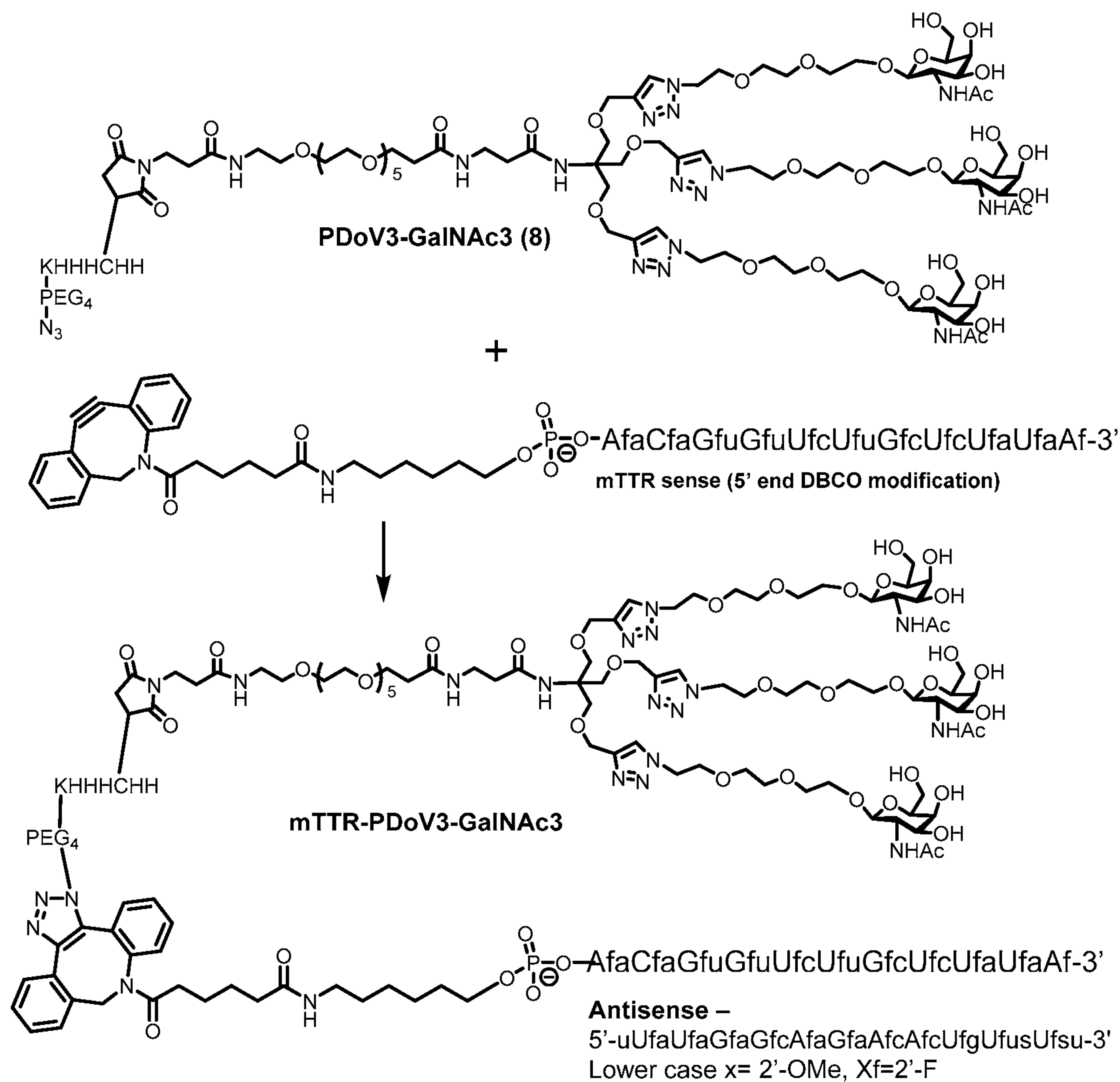
the crude material was purified by Glen Gel PaK column chromatograph to provide the pure product sense ApoB100-PDoV3-GalNAc3 compound 12 (0.66 mg, yield 88%). The HPLC was performed on PA200 ion exchange column using phosphate buffer at pH=11, Rt= 13.015 min., purity >85%. MS (ESI, positive mode) Calc. 10911.66 for Formula: C₃₈₈H₅₁₀N₁₂₅O₂₀₈P₂₂S. Found: 10929.3 [M+H₂O]. After performing the 1:1 annealing (95°C for 5 min, cool down by around 1°C/min to room temperature, then store under -20 °C) with the ApoB100 antisense strand it provided the final conjugate duplex ApoB100-PDoV1-GalNAc3 (12). The dye Alex-647 labeled ApoB100-PDoV1-GalNAc3 was prepared by replacing the ApoB100 antisense with the dye labeled antisense ApoB100-Alexa647 (5'-uuuGfTaaucgucgauAfcccugcucg-Alexia647-3'). The product was characterized by HPLC and MS. The analytical data were all consistent with the expected structure.

Example 12.Synthesis and characterization of ApoB100-PDoV3-GalNAc3 15.

- 5 **PDoV3-GalNAc3 8** (150 μg , 0.148 μmol) was added to the solution of 5'-DBCO-C6HN-APOB100 sense strand (230.7 μg , 0.0319 μmol) in RNase free water (200 μL). The mixture was stirred at 25 $^{\circ}\text{C}$ for 3 hours. After solvent was removed by lyophilization, the crude material was purified by Glen Gel Pak column to provide the pure product **sense ApoB100-PDoV3-GalNAc3** compound **15** (298 μg , yield 90%). The HPLC was performed on PA200
- 10 ion exchange column using phosphate buffer at pH=11, Rt= 12.002 min., purity >89%. 1:1 annealing (95 $^{\circ}\text{C}$ for 5min, cool down by around 1 $^{\circ}\text{C}/\text{min}$ to room temperature, then store under -20 $^{\circ}\text{C}$) with the antisense provided the final conjugate duplex **ApoB100-PDoV3-GalNAc3**. The dye **Alexa-647** labeled **ApoB100-PDoV1-GalNAc3** was prepared by replacing the **ApoB100** antisense with the dye labeled antisense ApoB100-Alexa647 (5'-uuuGfTaaucgucgauAfcccugcucg-Alexia647-3'). The product was characterized by HPLC
- 15 and MS. The analytical data were all consistent with the expected structure.

Example 13.Synthesis and characterization of mTTR1-PDoV2-GalNAc3 (16):

mTTR1-PDoV2-GalNAc3 was prepared by following a similar procedure as with synthesis
 5 of **mTTR1-PDoV3-GalNAc3** by using PDoV2-peg6-GalNAc **6** (0.547 mg, 0.1374 μmol)
 and DBCO-labeled **mTTR1 sense strand** (0.5 mg, 0.0687 μmol) in DMSO/water solvent.
 The crude material was purified by gel permeation column chromatograph G25 column to
 provide the pure product. The yield was about 78%. After 1:1 annealing (95 °C for 5min,
 cool down by around 1 °C/min to room temperature, then store under -20 °C) with the
 10 antisense strand it provided the final conjugate duplex **mTTR1-PDoV2-GalNAc3 (16)**.

Example 14.Synthesis and characterization of mTTR1-PDoV3-GalNAc3 17:

PDoV3-GalNAc3 8 (996 μ g, 0.317 μ mol) in DMSO (99.6 μ L) was added to a solution of **mTTR1-sense-5'-DBCO** (1.54 mg, 0.212 μ mol) in RNase free water (100 μ L). The resultant mixture was stirred at 25°C for 2 hours. After solvent was removed under reduced pressure, the crude material was purified by gel permeation column chromatograph PD-10 column to provide the pure product **sense mTTR1-PDoV3-GalNAc3** compound **17** (mg, yield 85%). The HPLC was performed on PA200 ion exchange column using phosphate buffer at pH=11, Rt= 14.744 min., purity >85%. 1:1 annealing (95 °C for 5min, cool down by around 1 °C/min to room temperature, then store under -20 °C) with the antisense strand provided the final conjugate duplex **mTTR1-PDoV3-GalNAc3**.

Example 15.In vitro study of conjugates of APOB100-PDoVn-GalNAc3:

Alexa 647 labeled siRNA-GalNAc conjugates (**ApoB100-PDoV1-GalNAc3-Alexa647**, **ApoB100-PDoV2-GalNAc3-Alexa647**, **ApoB100-PDoV2-GalNAc3-Alexa647**) were used in the *in vitro* cell uptake experiment. Lipofectamine-delivered Alexa 647 labeled siRNA was used as a control. HepG2 cells and Huh7 cells with ASGPR overexpression were used to compare with the wild type cells in the transfection experiment. 5000 Cells were plated in a 96 well plate overnight. Cells were transfected with 50nM siRNA for 2h, 24, and 48 hours. Nuclei were stained with Hoechst dye and cells were imaged in Cytation imaging station in PBS. Images were taken at x20 magnification. The data obtained showed that HepG2 cells overexpressing ASGPR showed much greater uptake of fluorescent labeled material than wild type HepG2 cells lacking overexpressed ASGPR.

Huh7 cells overexpressing ASGPR also showed an increased uptake compared with Huh7 cells not overexpressing ASGPR receptors but the labeling was not as intense as in wild type HepG2 cells.

PDOV1 and PDOV2 both demonstrated delivery of labeled siRNA to HepG2 cells overexpressing ASGPR but little or no labeling of wild type cells. In the absence of GalNAc on the constructs we did not see any uptake into the ASGPR overexpressing cells.

In a subsequent experiment we examined delivery with PDOV1, 2 and 3 variations in Huh7 overexpressing ASGPR. PDoV2 and 3 showed some specificity of uptake into these cells compared with Wild type cells. PDoV1 also showed some delivery to these cells but was not as great as PDoV2 and PDoV3. PDoV2 showed significant uptake after 24h exposure.

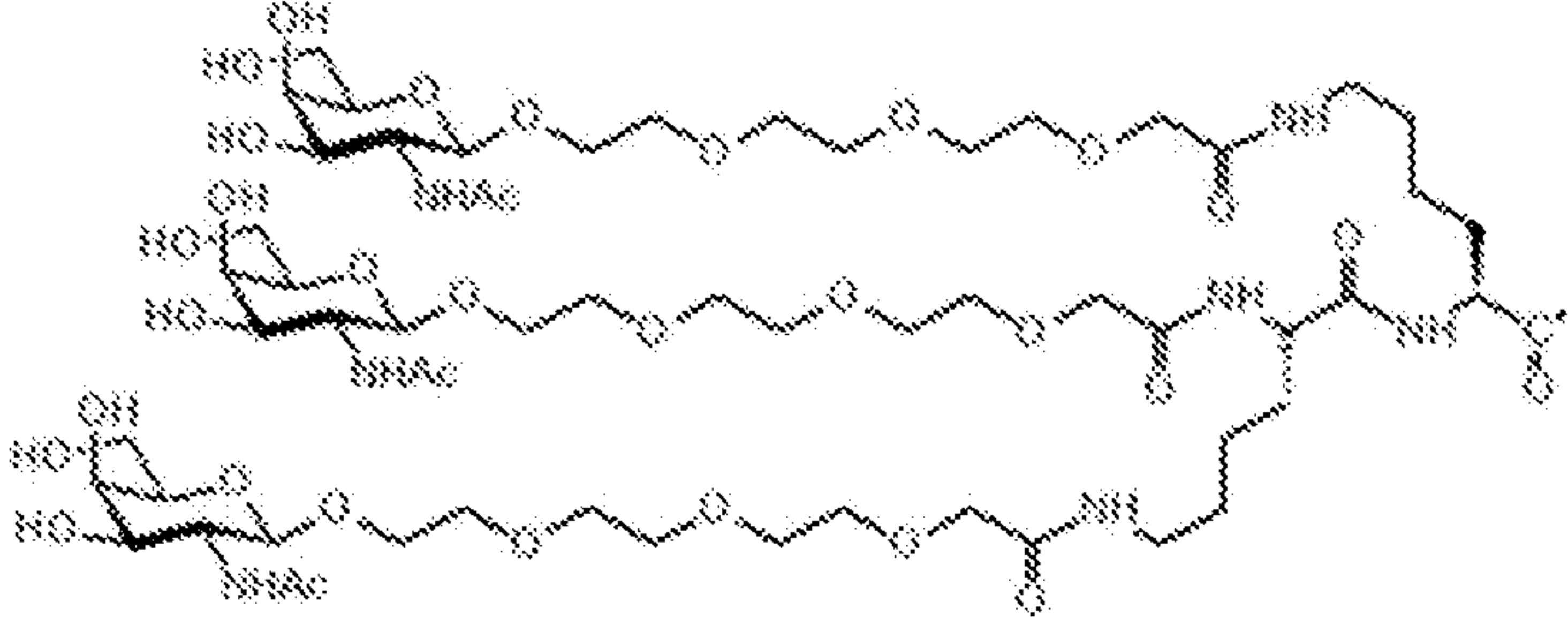
Example 16.In vitro study of mTTR-PDoVx-GalNAc in primary mouse hepatocytes:

Dose-response screens for GalNAc-conjugated siRNAs (mTTR1-PDoV3-GalNAc3, mTTR1-PDoV2-GalNAc3, mTTR2-GalNAc3 positive control, mTTR2-GalNAc3 positive control) were performed using primary mouse hepatocytes for target TTR. The cell density was 40000 cells/96 well. The concentration was from: highest at 1 μ M in 5-fold dilutions to lowest at 3 pM. Cells were transfected by direct incubation for 72 hours. The data was read out by bDNA assay. The data was analyzed by XLfit software. TTR signal normalized to gapdh, mean of quadruplicates, mock-treated cells set = 1, XLfit for calculation of IC50 values. The primary

hepatocytes used were purchased from Primacyt, Germany (lot MH181219), derived from CD1 mice, and treated with siRNA right after thawing.

The two siRNAs were both conjugated to trivalent GalNAc clusters at the 3' end (mTTR2 siRNA sequence was a fully modified sequence and 4 more additional PS linkages modification on the oligonucleotides than mTTR1. (Nair, *et al.*, *J. Am. Chem. Soc.* 136:16958–16961 (2014). Aha1-GaNAc3 positive control was used as a standard control, targeted to the housekeeper ahsa1. This was used as both negative and positive control, hybridizing the cell lysates either to the screening target or to an ahsa1-specific probe. Table e1 lists the sequences of the 2 control siRNAs and the structure of the GalNAc cluster.

Table e1. Control siRNA sequence and targets.

conjugates	target	sequence
mTTR2-GaNAc3 positive control	mouse TTR	Sense: 5'-AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf(NHC6)(GalNAc3)-3' Antisense: 5'-usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu-3'
Aha1-GaNAc3 positive control	AHSA1	Sense: 5'-uscsUfcGfuGfgCfcUfuAfaUfgAfaAf(NHC6)(GalNAc3)-3' Antisense: 5'-UfsUfsuCfaUfuAfaGfgCfcAfcGfaGfasusu-3'
Trivalent GalNAc3 ligand on the control conjugate		

10

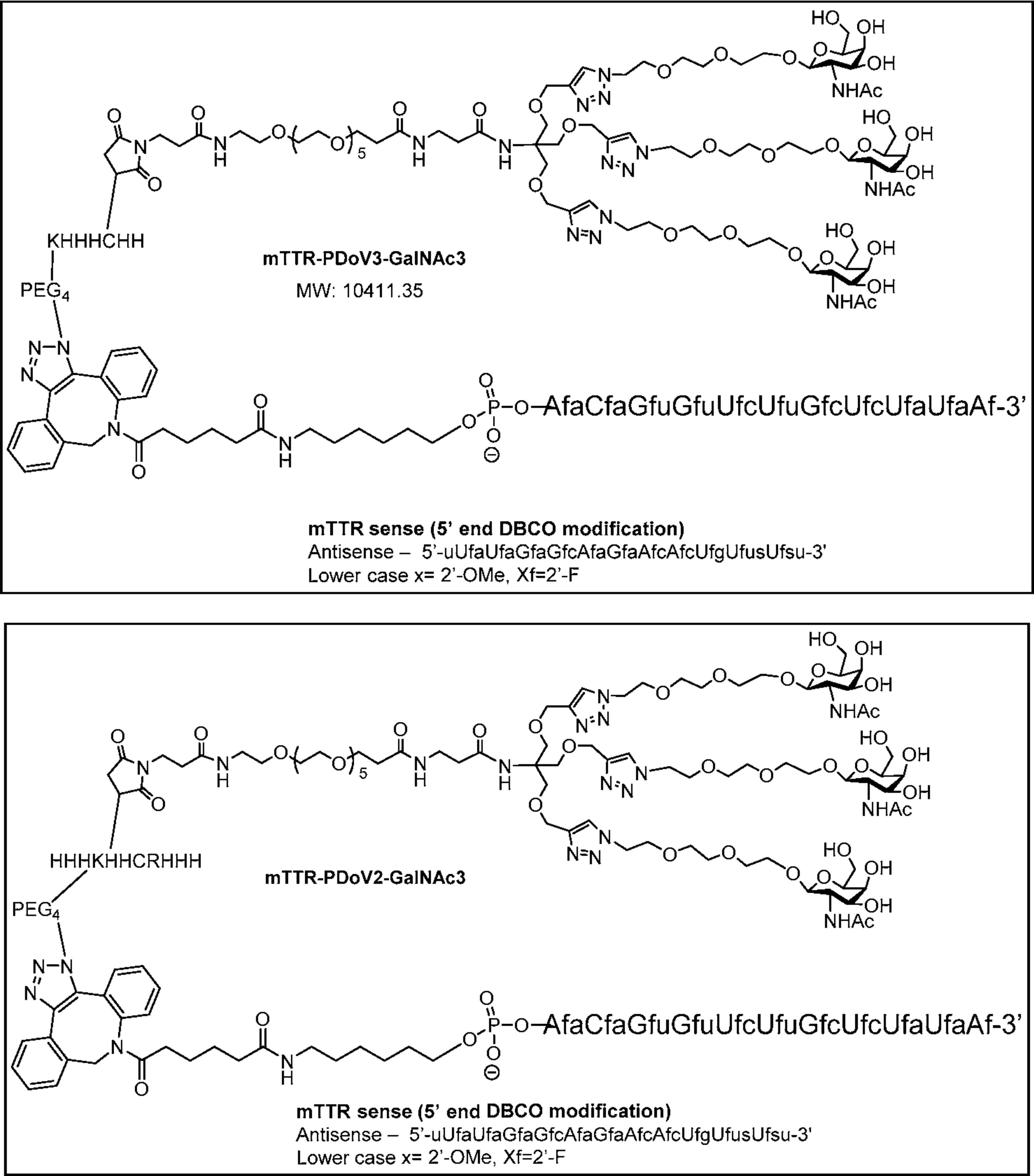


Table e2. Dose-response summary for GalNAc-conjugated siRNAs in vitro screening.

Compound	IC ₅₀ (μM)	IC ₈₀ (μM)	Max. KD (%)
mTTR1-PDoV3-GalNAc3	0.0018	0.0048	97%
mTTR1-PDoV2-GalNAc3	0.0026	0.0071	97%
mTTR2-GalNAc3 positive control	0.0001	0.0003	96%
aha1-GalNAc3 positive control	0.0015	0.0059	96%

5 **Conjugate of mTTR1-PDoV3-GalNAc3 in vitro serious dilution data** – see Figure 15A: the IC₅₀=1.82 nM is comparable with the literature report of IC₅₀=1.39 nM for the same siRNA sequence 21 and chemical modification on the oligonucleotides (Nair *et al.*, *supra.*) Figure 15B shows *in vitro*, serial dilution data for the mTTR1-PDoV2-GalNAc3 conjugate. Figure 15C shows serial dilution data for the mTTR2-GalNAc3 positive control conjugate in vitro

10 serial dilution data: mTTR2 siRNA sequence is a fully modified sequence and has 4 more

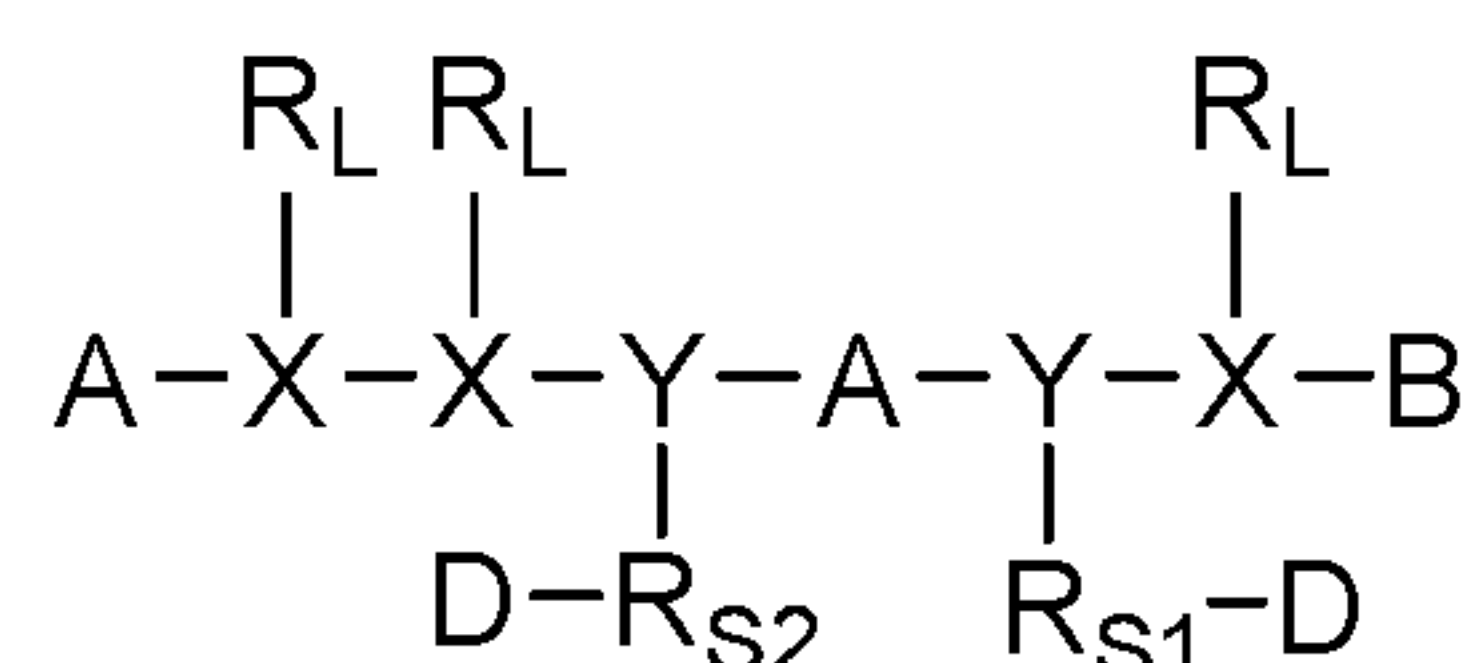
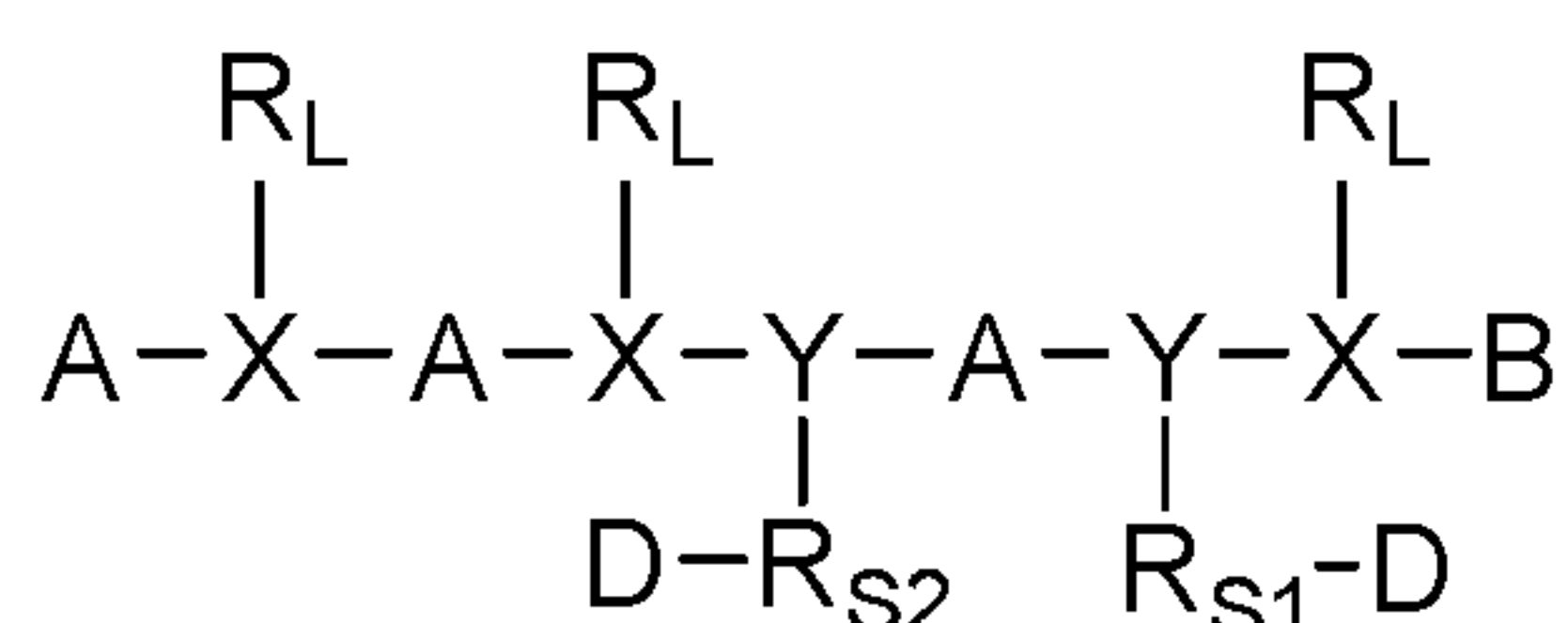
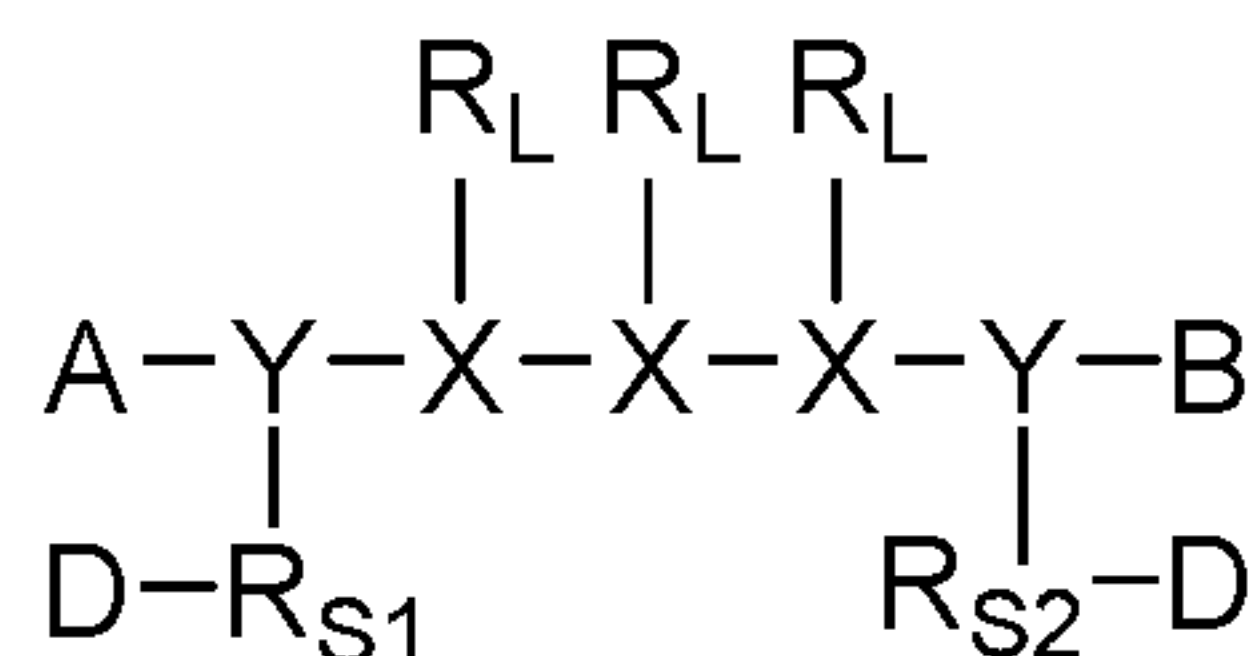
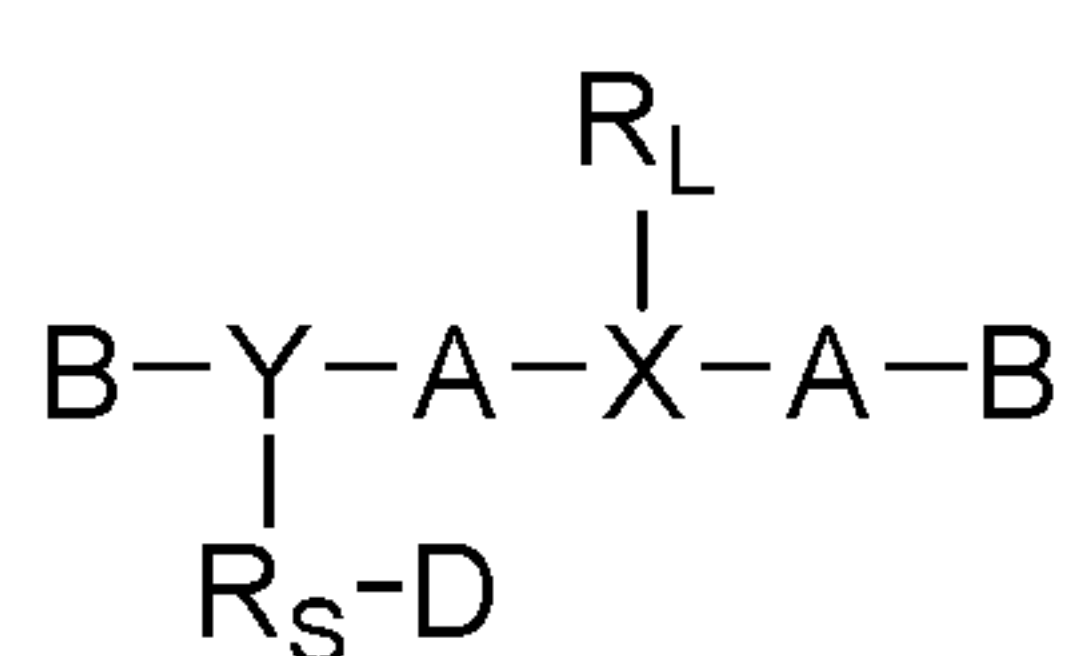
additional PS linkages modification on the oligonucleotides (Nair, *supra.*) Figure 15D shows in vitro serial dilution data for the Aha1-GalNAc3 positive control conjugate.

5 All publications identified herein, including issued patents and published patent applications, and all database entries identified by url addresses or accession numbers are incorporated herein by reference in their entirety.

Although this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied without departing from the basic principles of
10 the invention.

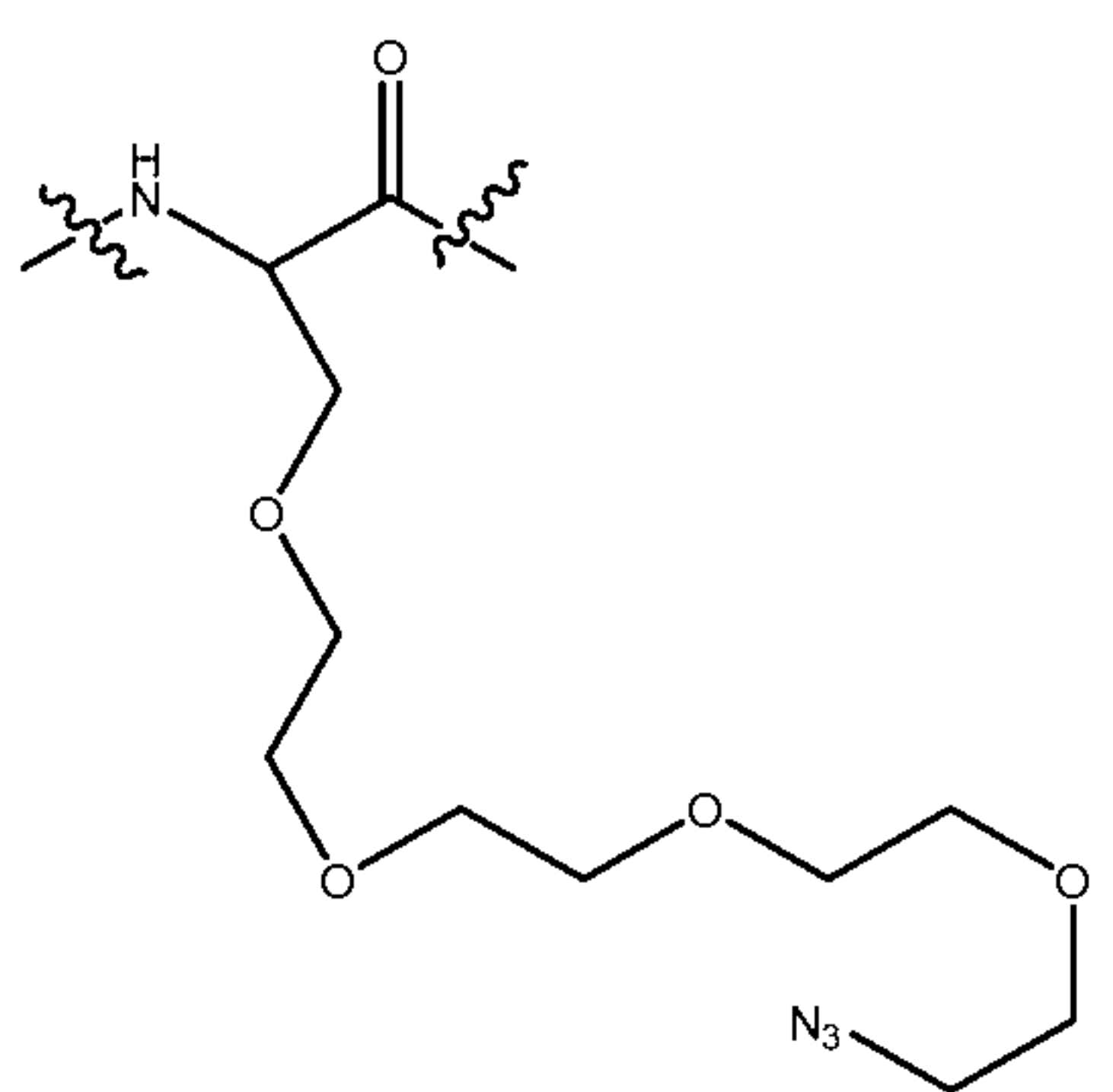
CLAIMS

1. A chemical construct comprising a Peptide Docking Vehicle (PDoV) covalently linked to (a) a targeting moiety, and (b) a first therapeutic oligonucleotide.
2. The construct of claim 1, wherein the construct comprises a second therapeutic oligonucleotide that is the same as, or different from, the first therapeutic oligonucleotide.
3. The construct of any preceding claim wherein the PDoV comprises multiple repeating units of histidine and lysine.
4. The construct of any preceding claim wherein the targeting moiety binds to the asialoglycoprotein receptor.
5. The construct of any one of claims 1-4, wherein the oligonucleotide comprises an siRNA, an antisense oligonucleotide, an miRNA, an saRNA, an shRNA, an aptamer, an RNAzyme, an DNAzyme, a decoy oligonucleotide, or a CpG motif.
6. The construct of claim 5, wherein said therapeutic oligonucleotide is an siRNA or miRNA molecule.
7. The construct of any preceding claim, wherein the PDoV construct comprises an endosomal release motif that comprises at least two targeting moieties that are the same or different and/or at least two therapeutic oligonucleotides that are the same or different.
8. The construct of any preceding claim having one of the structures shown below, wherein: type X sites are used to conjugate the targeting ligands; Type Y sites are used to conjugate the oligonucleotide, and X and Y can be the same or different; A is a peptide sequence of H, K, R, HH, HHH, HHHH, HHK, HHHK or any other endosomal releasing short peptide containing up to about 5 amino acids; B is a peptide sequence of H, K, R, HH, HHH, HHHH, HHK, HHHK, or any other short peptide containing up to about 5 amino acids, any other amino acid or combination of amino acids with a linker; D is an oligonucleotide; R_L is ligand; and R_S is a linker to the oligonucleotide.

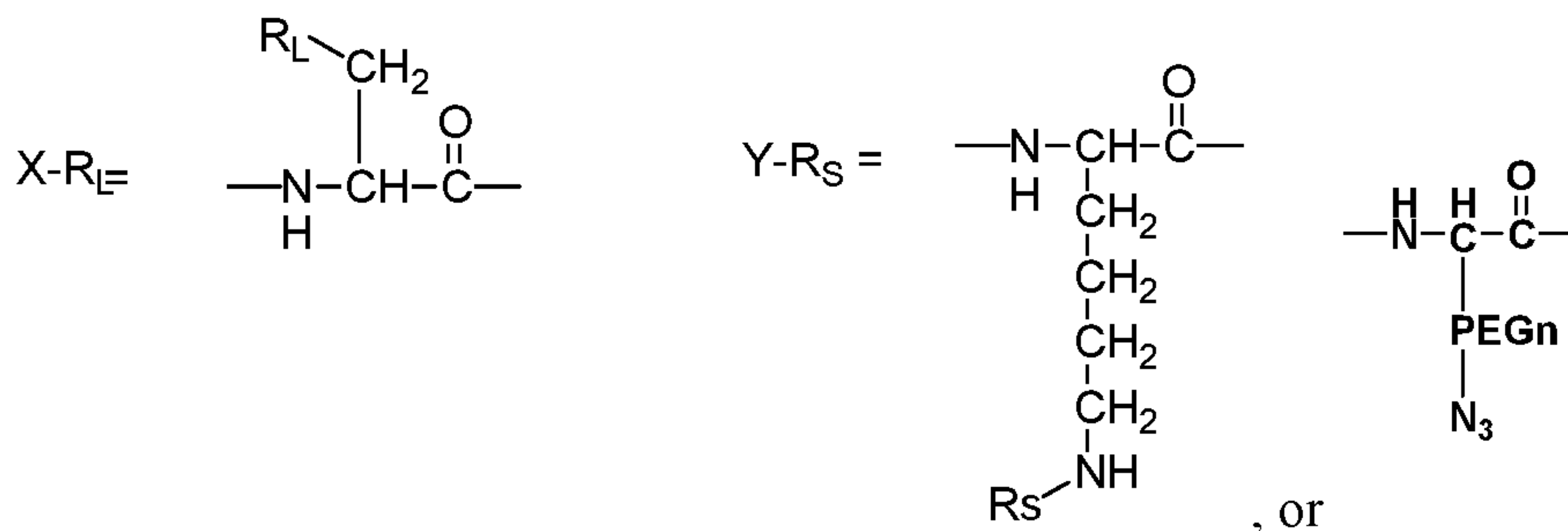
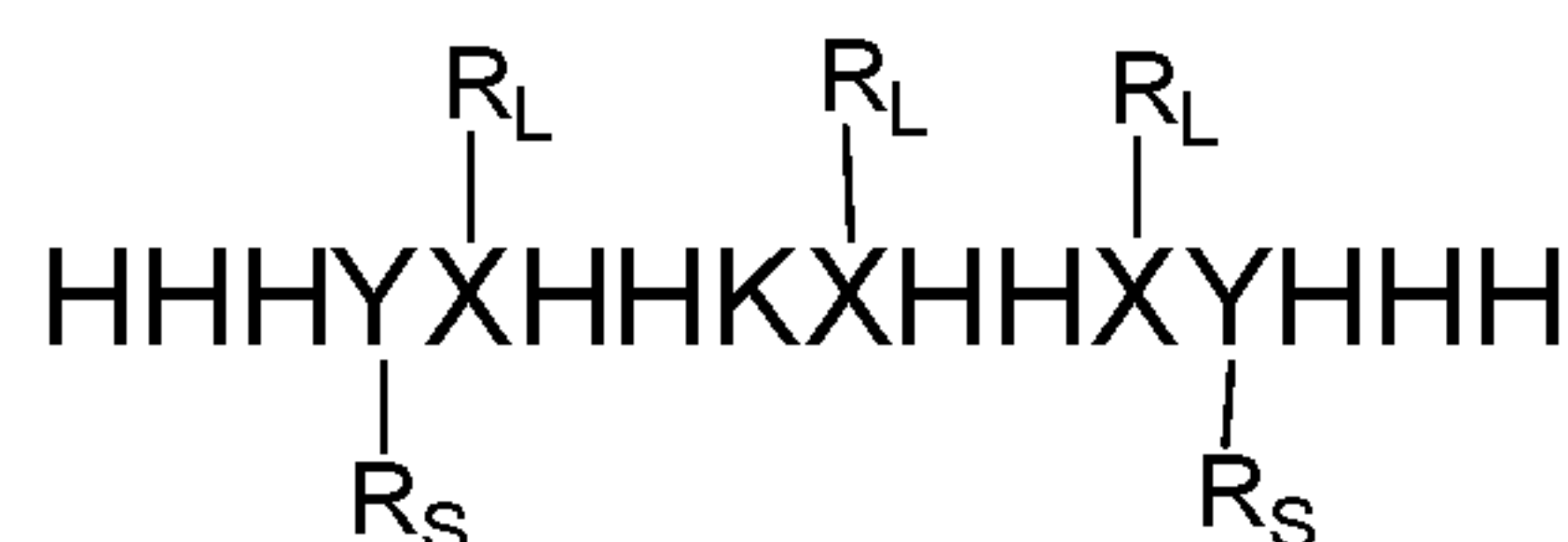


9. The construct of claim 8, wherein D is an siRNA, mRNA, or an aptamer.

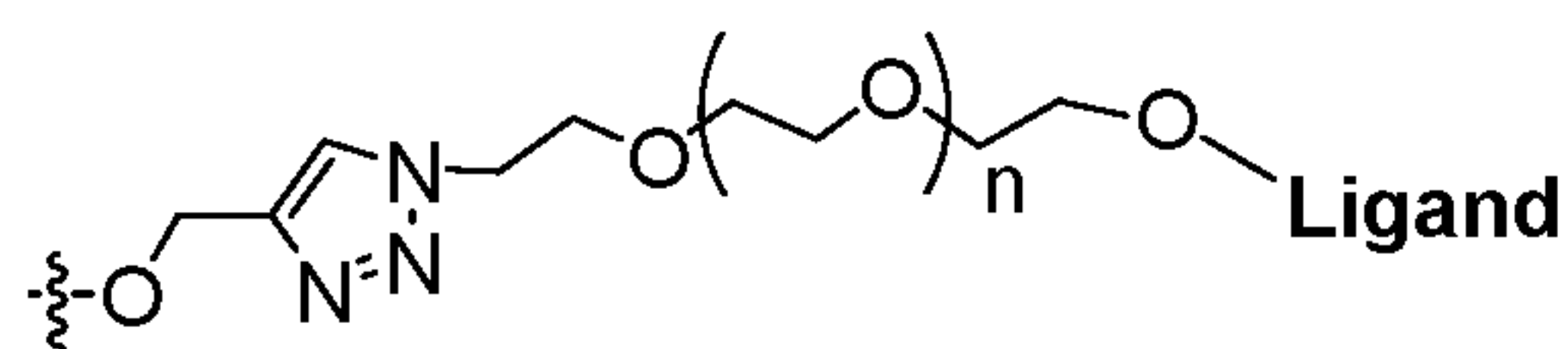
10. The construct of any preceding claim, wherein the PDoV peptide construct has a structure comprising an amino acid sequence selected from the group consisting of KHHHCKH, HKHHHCKH, HHKHHHCKH, HHHKHHHKCHHHKHHH, HHHKHHCKHHH, HHHKHHHCRHHH, HKHHCKH, HKHCH, HKHCKH, HKHC, HHHK(S)HHCKHHH, and HHK(S)HHKCHH(S)HHH, wherein R_L is linked to the side chain of the cysteine residue and R_S is linked to the side chain of the lysine residue, and wherein (S) is:



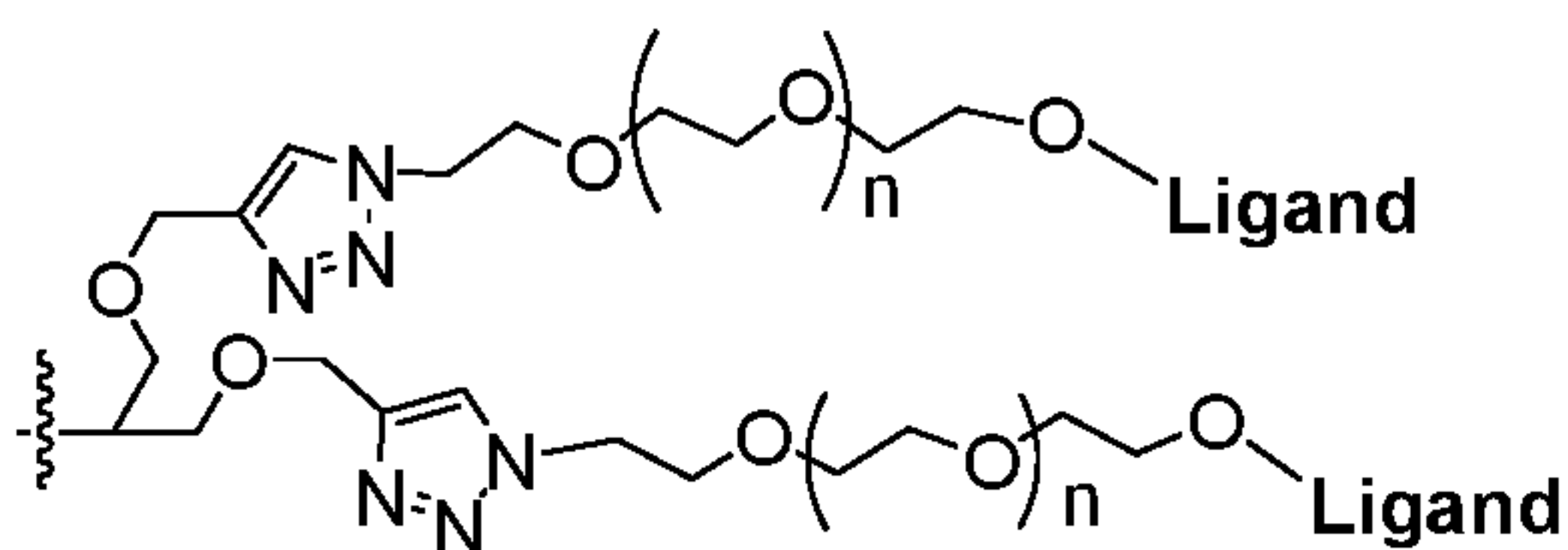
10. The construct of any one of claims 1-9, wherein the PDoV peptide construct has the structure below, and in which three of the ligands are conjugated individually on the peptide.



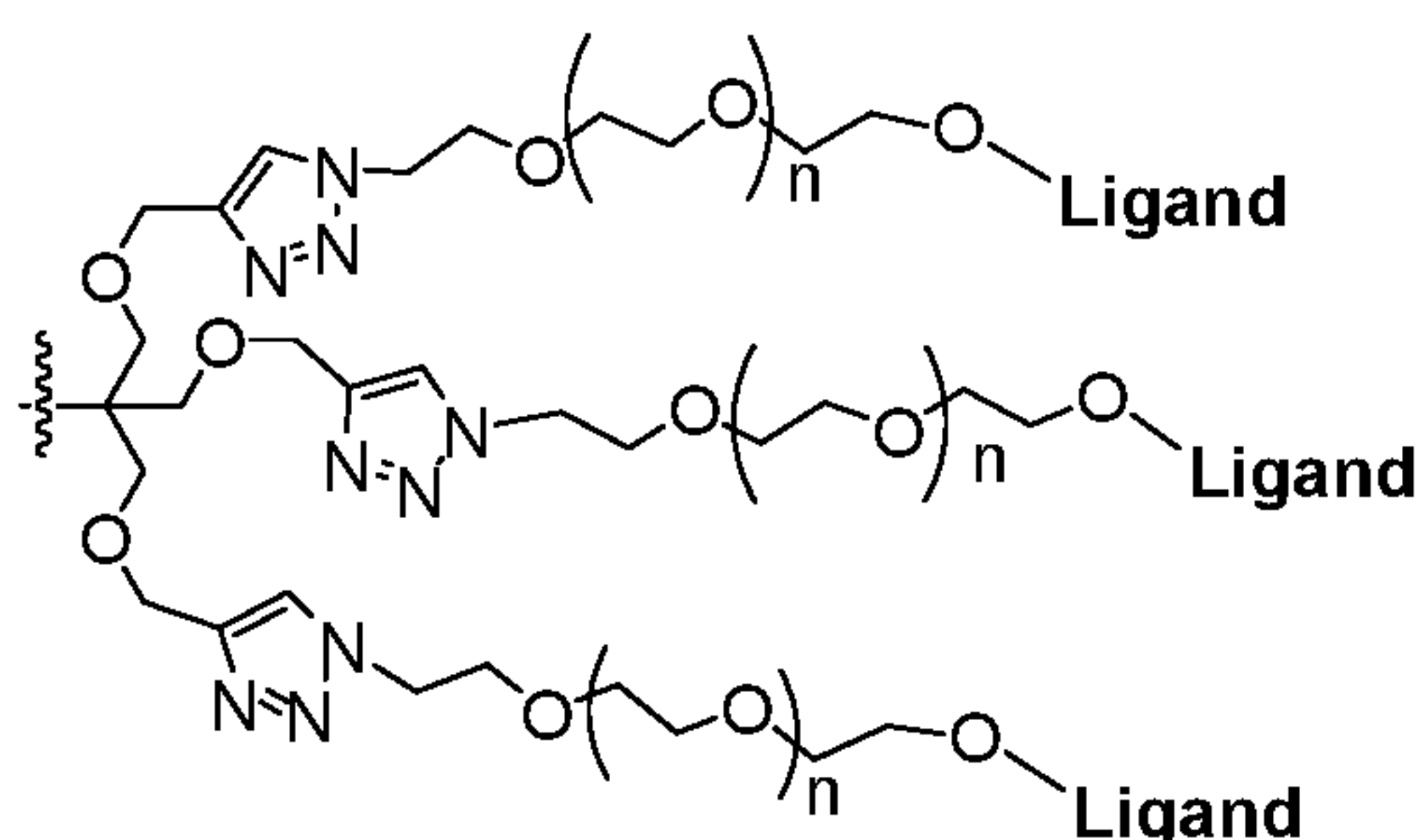
11. The construct of any preceding claim wherein the ligand moiety R_L comprises linker-1, wherein linker-1 comprises one of the following structures:



, wherein n is 1, 2, or 3 and is connected to the bridge through a 1, 5-triazol ring with an OCH₂ unit; or



, wherein n is 1, 2, or 3 and is connected to the bridge through a 1, 5-triazol ring with a CH₂OCH₂ unit; or



, wherein n is 1, 2, or 3 and is connected to the bridge through a 1, 5-triazol ring with a CH₂OCH₂ unit.

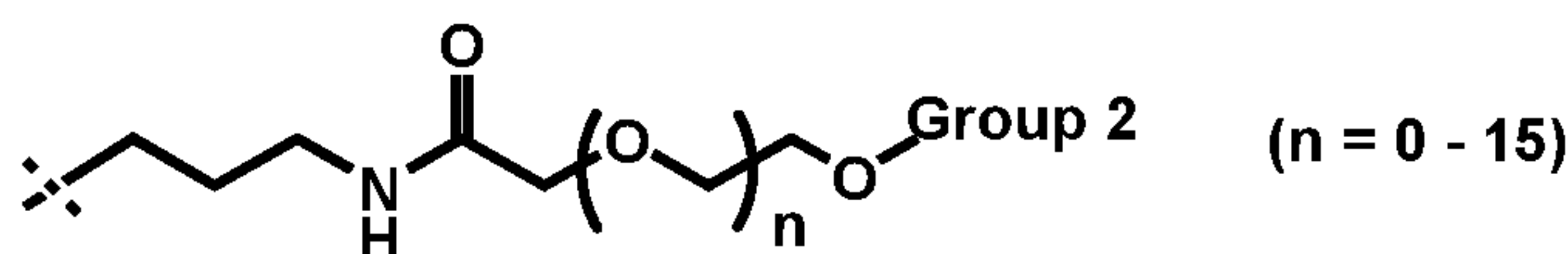
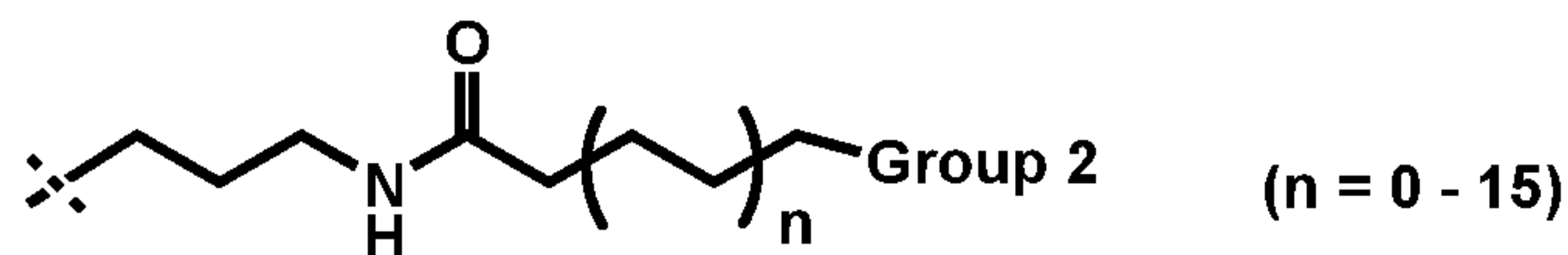
12. The construct according to any of claims 1-10, wherein the linker between the ligand(s) attached to the PDoV peptide comprises a polyethylene glycol chain – (CH₂CH₂O)_n–, or –(CH₂CH₂)_n– chain, wherein n is an integer from 2-15.

13. The construct of any preceding claim where the ligand R_L is linked to the PDoV peptide via a linker that comprises a thioether, an amide, or a triazole linkage.

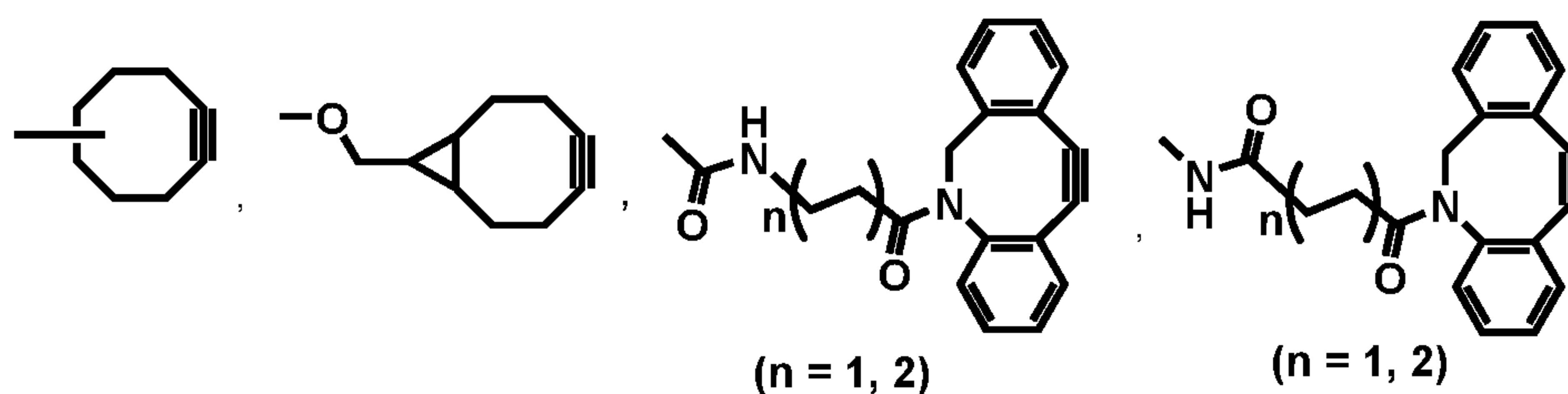
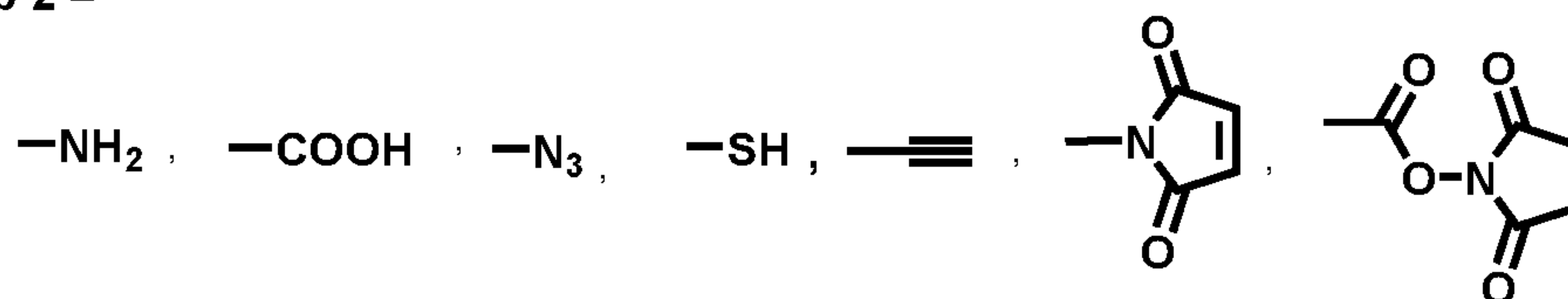
14. The construct of claim 1, wherein the oligonucleotide is linked via a moiety having the structure R_s-linker2-oligonucleotide, wherein linker 2 is an aliphatic chain, a polyethylene glycol chain, a hydrophobic lipid chain or a hydrophilic chain. The group 2 at the end site is the reactive site for the chemical conjugation with the siRNA end.

15. The construct of claim 15 wherein:

Linker 2 =



Group 2 =



16. The construct of claim 1, wherein the oligonucleotide is an RNAi molecule that is single-stranded oligonucleotide with a length of 10-29.

17. The construct of claim 6 wherein the oligonucleotide is an siRNA molecule comprising a duplex of two complimentary, single-stranded oligonucleotides each with a length between 10-29 bases or 19-27 bases.

18. The construct of claim 1 where the oligonucleotide comprises deoxyribonucleotides.

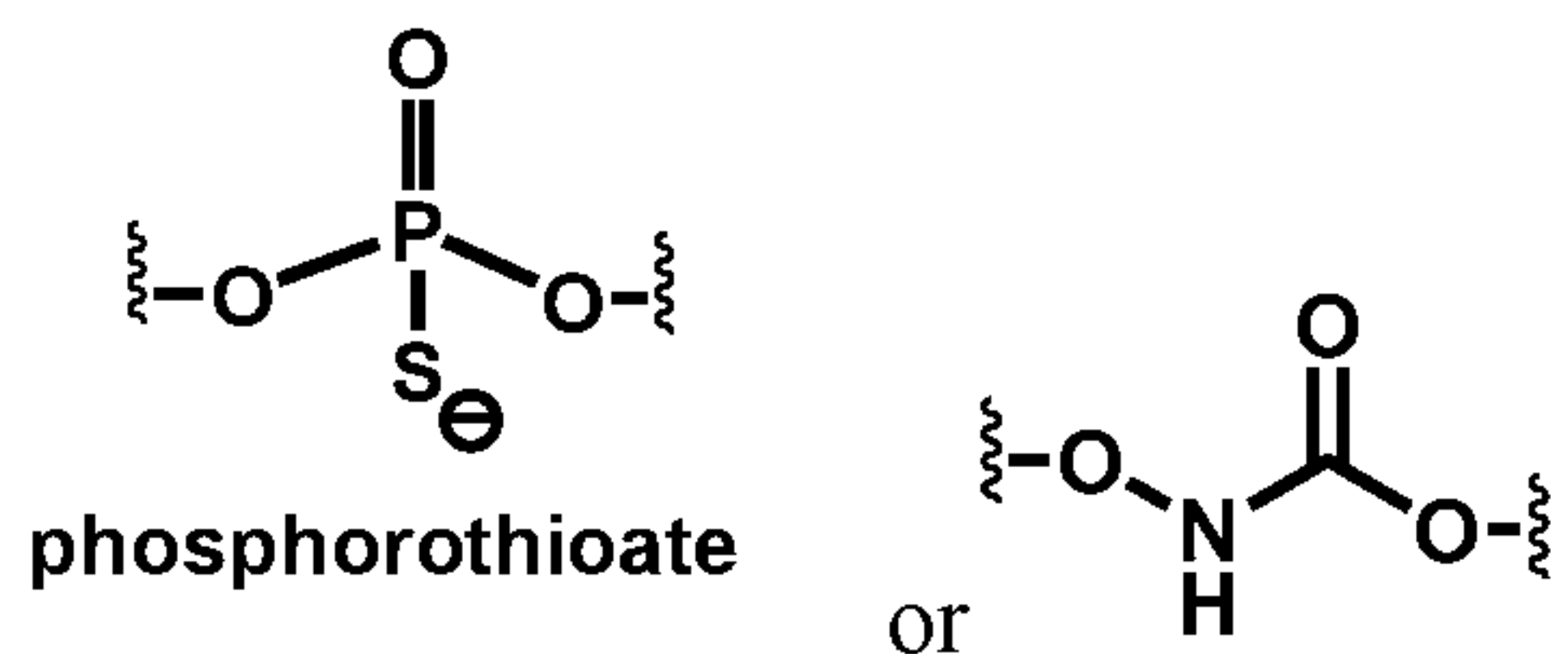
19. The construct of any one of claims 1-17, wherein the oligonucleotide comprises ribonucleotides.

20. The construct of any one of claims 1-19, wherein the nucleotide comprises deoxyribonucleotides and ribonucleotides.

21. The construct of any one of claims 1-10-, wherein the oligonucleotide comprises an siRNA molecule comprising at least one or more chemically modified nucleotides at the 2' position.

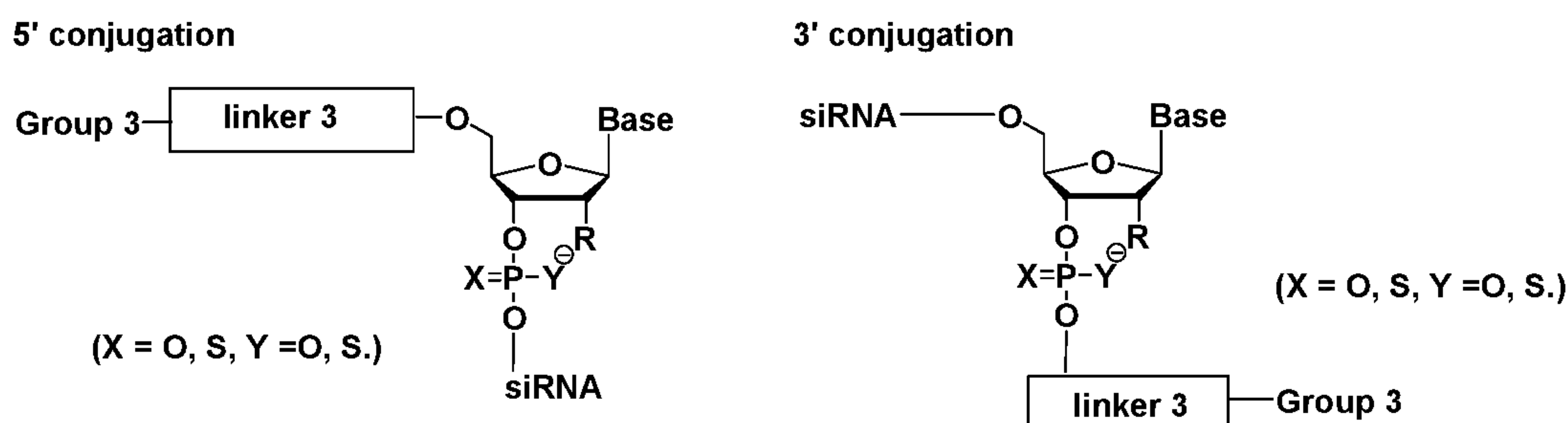
22. The construct of claim 28, wherein the chemically modified nucleotides comprise 2'-O-Methyl or 2'-Fluoro, 2'-O-methoxyethyl, 2'-O-allyl, or 2'-H, modified nucleotides.

23. The construct of any one of claims 1-22, wherein the oligonucleotide comprises an siRNA molecule comprising at least one chemically modified linkage that is



24. The construct of any preceding claim, wherein the siRNA or oligonucleotide is further chemically modified at position 5' or 3' with a linker 3, wherein Linker 3 contains a complimentary conjugation site group 3 which can covalently react with the linker 2 to link the ligand-PDoV and nucleotides.

25. The construct of claim 24, comprising a structure as shown below:



26. The construct of any preceding claim one of claims 1-32, wherein the targeting ligand is selected from the group consisting of N-acetyl-galactosamine (GalNAc), galactose, galactosamine, N-formal-galactosamine, N-propionyl-galactosamine, N-butanoylgalactosamine and aptamer.

27. The construct of claim 26, wherein the targeting ligand is N-acetyl-galactosamine (GalNAc).

28. The construct of claim 1, wherein the targeting ligand is a peptide selected from the group consisting of cyclic(c) RGD, APRPG, NGR, F3 peptide, CGKRK, LyP-1, iRGD (CRGDRCPDC), iNGR, T7 peptide (HAIYPRH), MMP2-cleavable octapeptide (GPLGIAGQ), CP15 (VHLGYAT), FSH (FSH-β, 33-53 amino acids, YTRDLVKDPPARPKIQKTCTF), LHRH (QHYSYkLRP), gastrin-releasing peptides (GRPs)

(CGGNHWAVGHLM), RVG (YTWMPENPRPGTPCDIFTNSRGKRASNG), FMDV20 peptide sequence (NAVPNLRGDLQVLAQKVART), and GLP or is folic acid.

29. The construct of claim 28, wherein the targeting ligand is RGD or GLP.

30. A pharmaceutical composition comprising the construct of any preceding claim and a pharmaceutically acceptable carrier.

31. The pharmaceutical composition of claim 30, wherein the pharmaceutically acceptable carrier comprises histidine -lysine rich polypeptide HKP, HKP(H), or lipofectamine.

32. The pharmaceutical composition of claim 30, wherein the pharmaceutically acceptable carrier comprises water and one or more of the group consisting of: potassium phosphate monobasic anhydrous NF, sodium chloride USP, sodium phosphate dibasic heptahydrate USP, glucose, and Phosphate Buffered Saline (PBS).

33. A method of delivering an oligonucleotide to a cell, comprising contacting the cell with a construct according to claim 1.

34. A method of delivering an oligonucleotide to a hepatocyte cell, comprising contacting the cell with a construct according to claim 4.

35. The method of claim 33 or claim 34, wherein the oligonucleotide is an siRNA molecule and is delivered to the mammalian cell or hepatocyte *in vivo*.

36. The method of any of claims 33-35 wherein the cell is a human cell.

37. The method of any one of claims 41-45, wherein the mammalian cell or hepatocyte is a human cell or hepatocyte.

38. A method of gene therapy in a mammal comprising administering a therapeutically effective amount of the construct of any of claims 1-29 or the composition of claims 30-32 to the mammal, wherein the mammal optionally is a human.

39. The construct of any one of claims 1-29, wherein the peptide comprises an amino acid sequence -HHK, -KHH, -HHH, -HHHK, -KHHH, -HHHHK, -KHHHH, -K(HHHK)_n (n= 1-8), -K(HHHHK)_n(HHHK)_m (n= 1-4, m=1-8), K(HHHHK)_n(HHHK)_m (n= 1-4, m=1-8).

40. The construct of claim 39, comprising a peptide with a sequence -K(HHHHK)_n(HHHK)_m, or K(HHHHK)_n(HHHK)_m-, wherein n is 1 and m is 3.

41. The construct of claim 52, wherein the peptide is an endosomal releasing peptide.

42. The construct of claim 52, wherein the peptide is a cell penetrating peptide.

43. A peptide with the formula $(H_nK_m)_oX_pY_q$, wherein H is histidine, K is lysine, X and Y are functional units selected from an amino acid and a linker, n is 1-10, m is 1-10, o is 1-10, p is 1-5, and q is 1-4.

44. The peptide of claim 43, wherein the peptide includes at least two conjugation sites for attaching a targeting ligand.

45. The peptide of claim 44, wherein the conjugation site is lysine or cysteine..

46. The peptide of any one of claims 43-45, wherein the peptide comprises at least two conjugation sites for attaching an oligonucleotide.

47. A chemical construct comprising the peptide of any one of claims 43-46 and at least one targeting ligand linked to the peptide at t least one conjugation site.

48. A chemical construct comprising the peptide of any one of claims 43-46 or the construct of claim 47 and at least one oligonucleotide linked to the peptide at at least one conjugation site.

49. The chemical construct of any of claims 1-29, wherein the targeting ligand isselected from the group consisting of cyclic(c) RGD, APRPG, NGR, F3 peptide, CGKRRK, LyP-1, iRGD (CRGDRCPCDC), iNGR, T7 peptide (HAIYPRH), MMP2-cleavable octapeptide (GPLGIAGQ), CP15 (VHLGYAT), FSH (FSH- β , 33-53 amino acids, YTRDLVKDPPARPKIQKTCTF), LHRH (QHTSYkcLRP), gastrin-releasing peptides (GRPs) (CGGNHWAVGHLM), RVG (YTWMPENPRPGTPCDIFTNSRGKRASNG), FMDV20 peptide sequence (NAVPNLRGDLQVLAQKVART), and GLP.

50. The chemical construct of any one of claims 1-29 or 49, wherein the oligonucleotide is selected from the group consisting of an siRNA, an antisense oligonucleotide, an miRNA, an saRNA, an shRNA, an aptamer, an RNAzyme, an DNAzyme, a decoy oligonucleotide, and a CpG motif.

51. The chemical construct of claim 50, wherein the oligonucleotide is an siRNA, an antisense oligonucleotide, or an miRNA.

52. A method for treating cancer in a mammal, comprising administering to the mammal a therapeutically effective amount of the construct of any one of claims 1-29 or 49-51.

53. The method of claim 52, wherein the cancer is a solid tumor.

54. The method of claim 52, wherein the cancer is selected from the group consisting of liver cancer, colon cancer, and pancreatic cancer.

55. The method of claim 54, wherein the cancer is liver cancer and is selected from the group consisting of hepatocellular carcinoma, metastatic colon cancer, and metastatic pancreatic cancer.

56. The method of any one of claims 52-55, wherein the mammal is a human.

57. The method of any one of claims 52-56, wherein the construct is injected directly into a tumor comprising the cancer cells.

Figure 1.

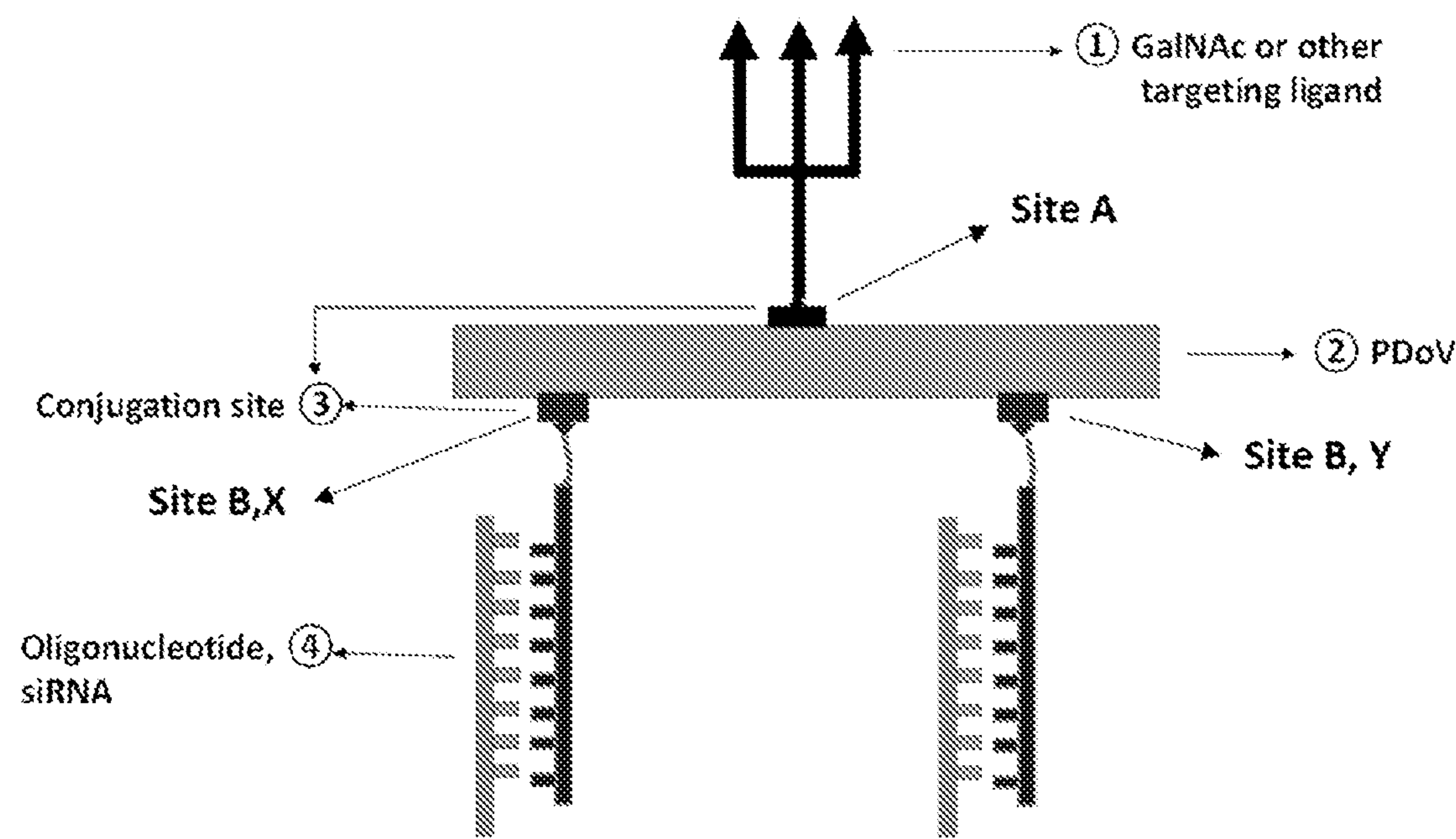


Figure 2.

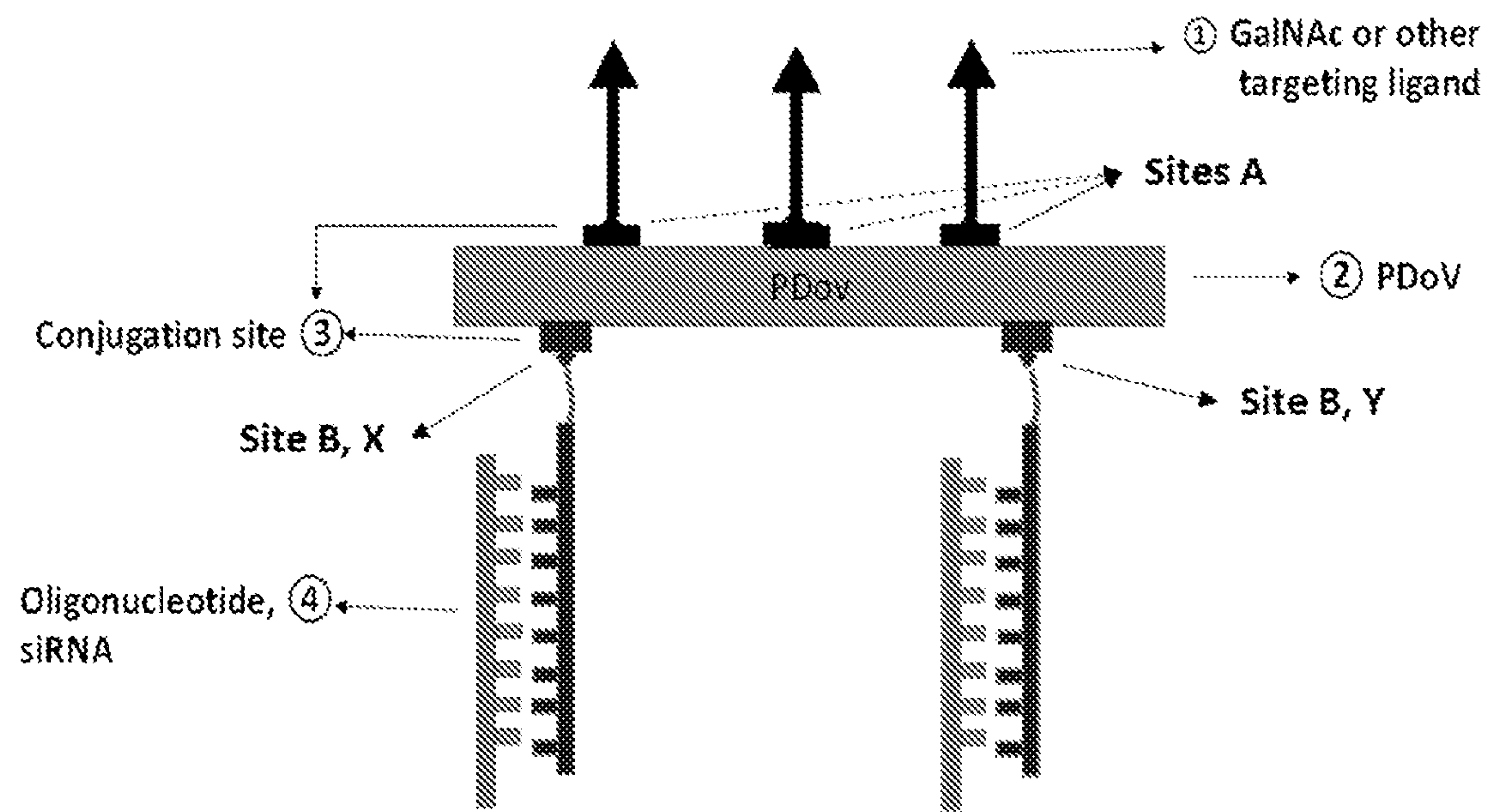


Figure 3.

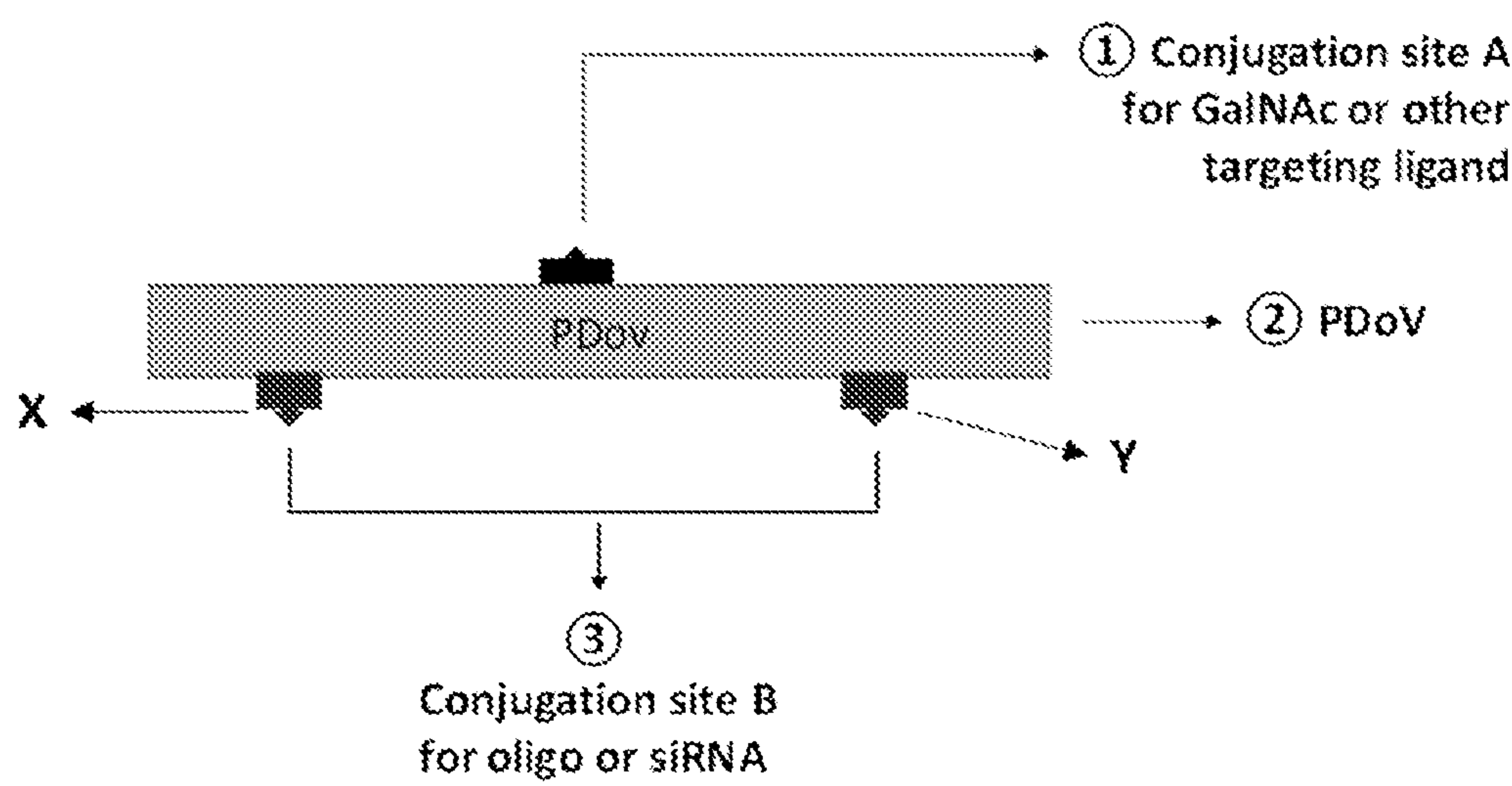


Figure 4.

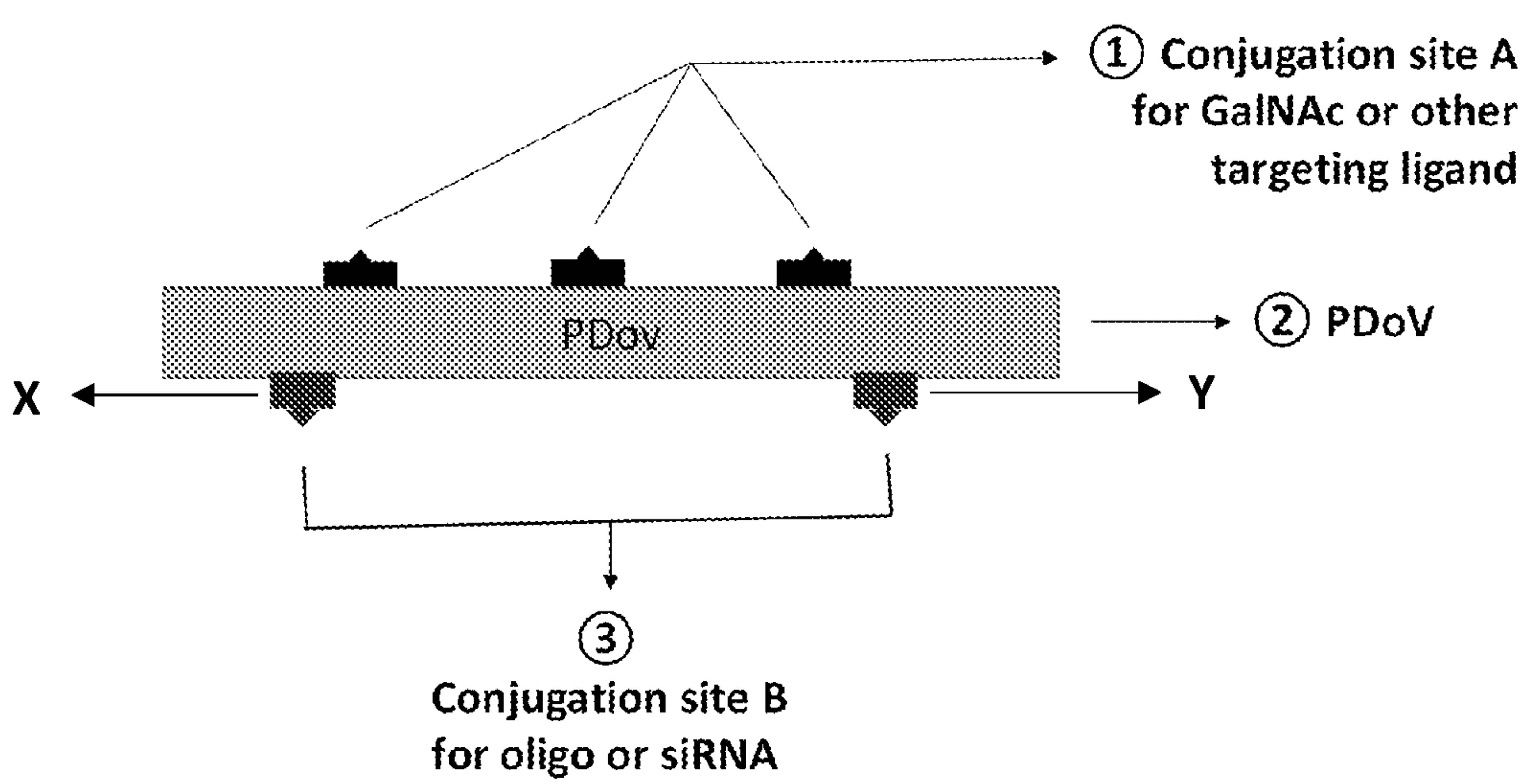
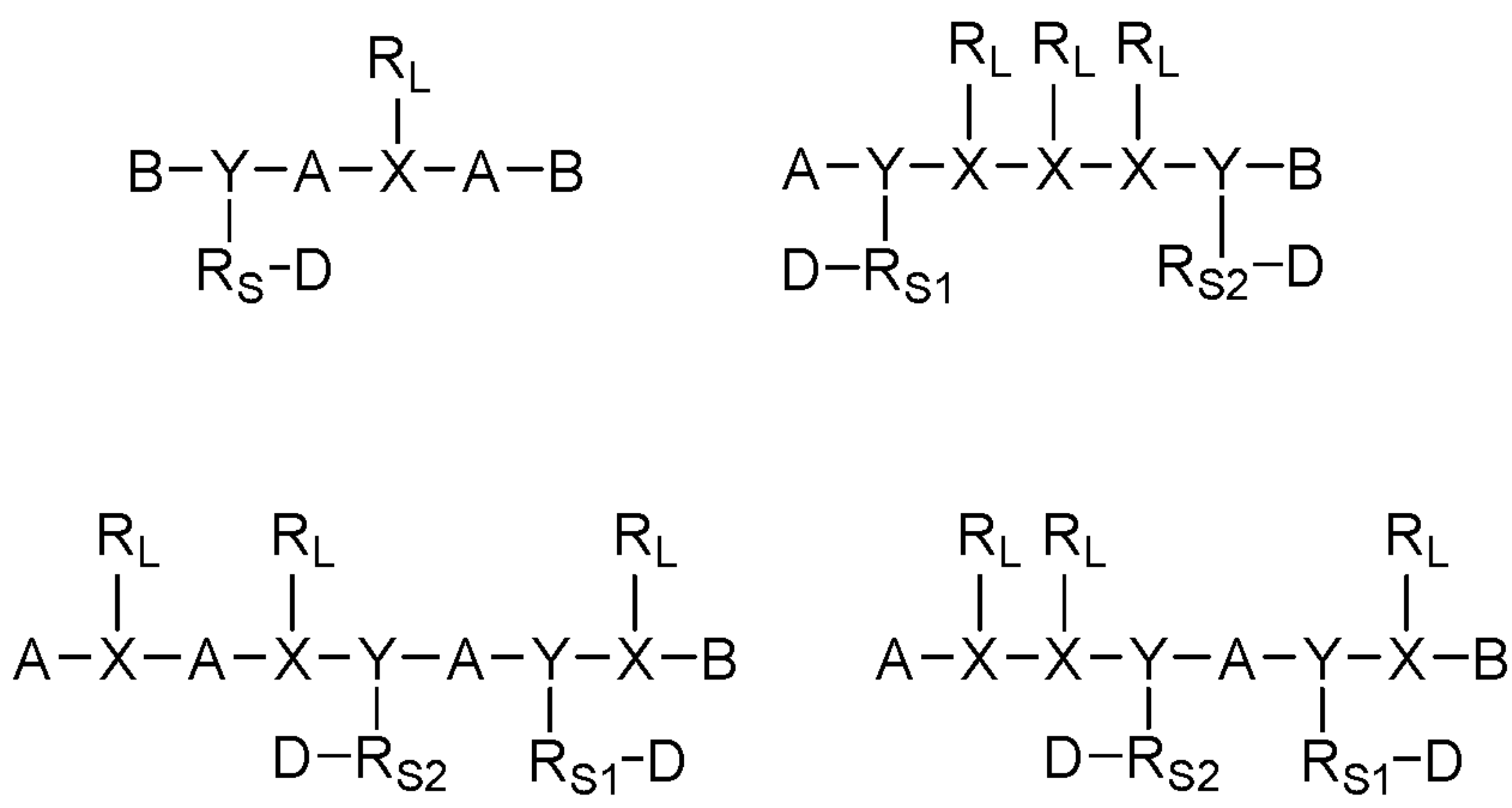
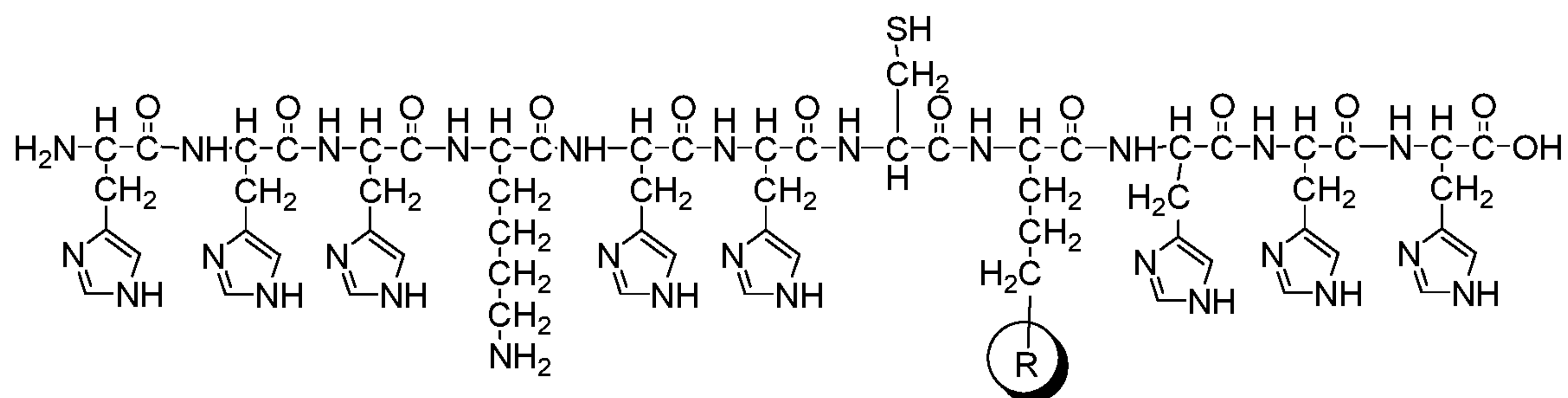


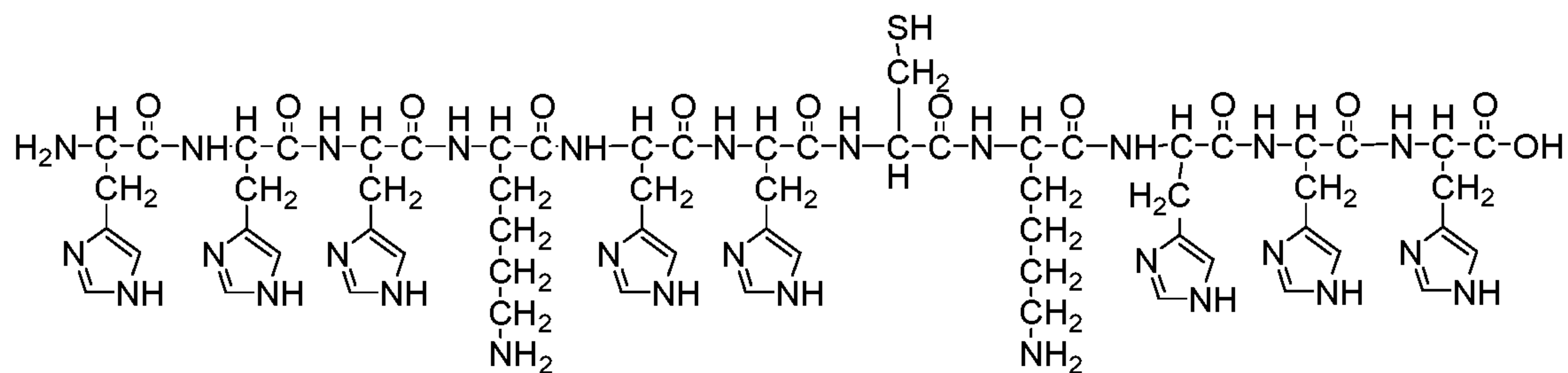
Figure 5.
Structure of the PDoV construct.



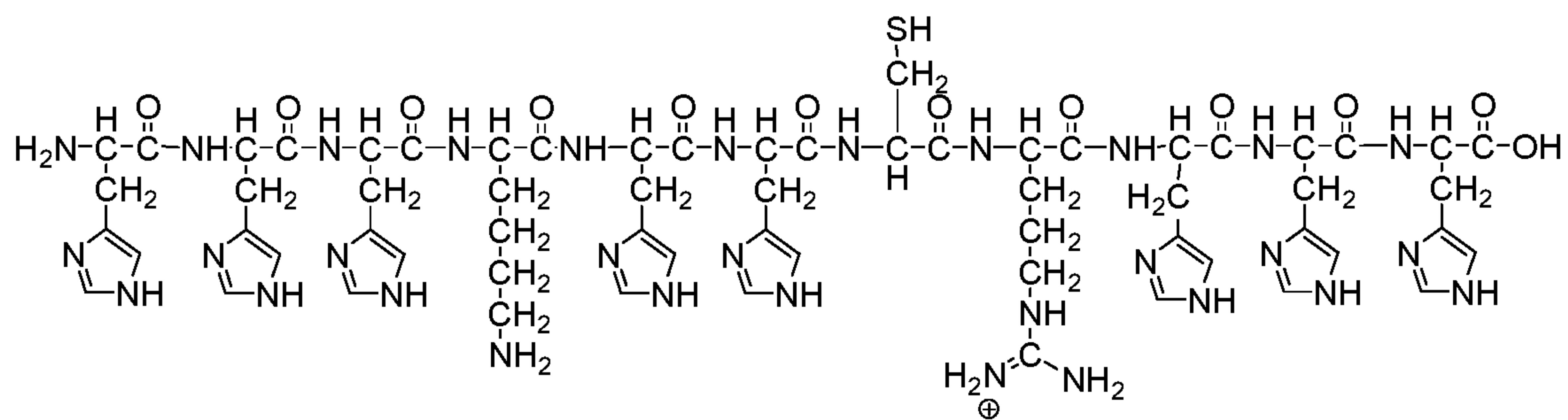
4/16

Figure 6.

,

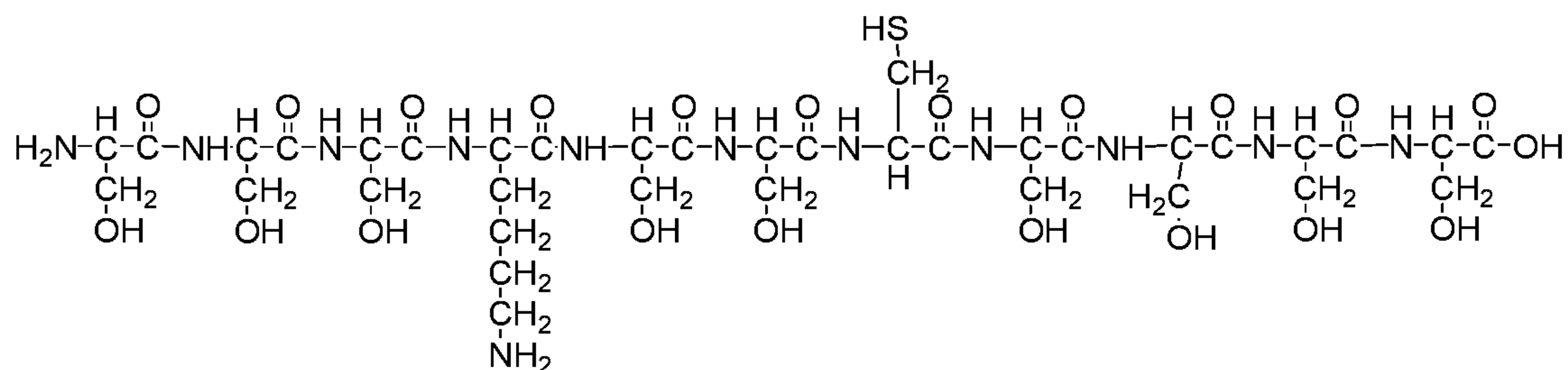
when R= CH₂NH₂ (PDoV1),

,

when R= NHC(NH₂)₂ (PDoV2):

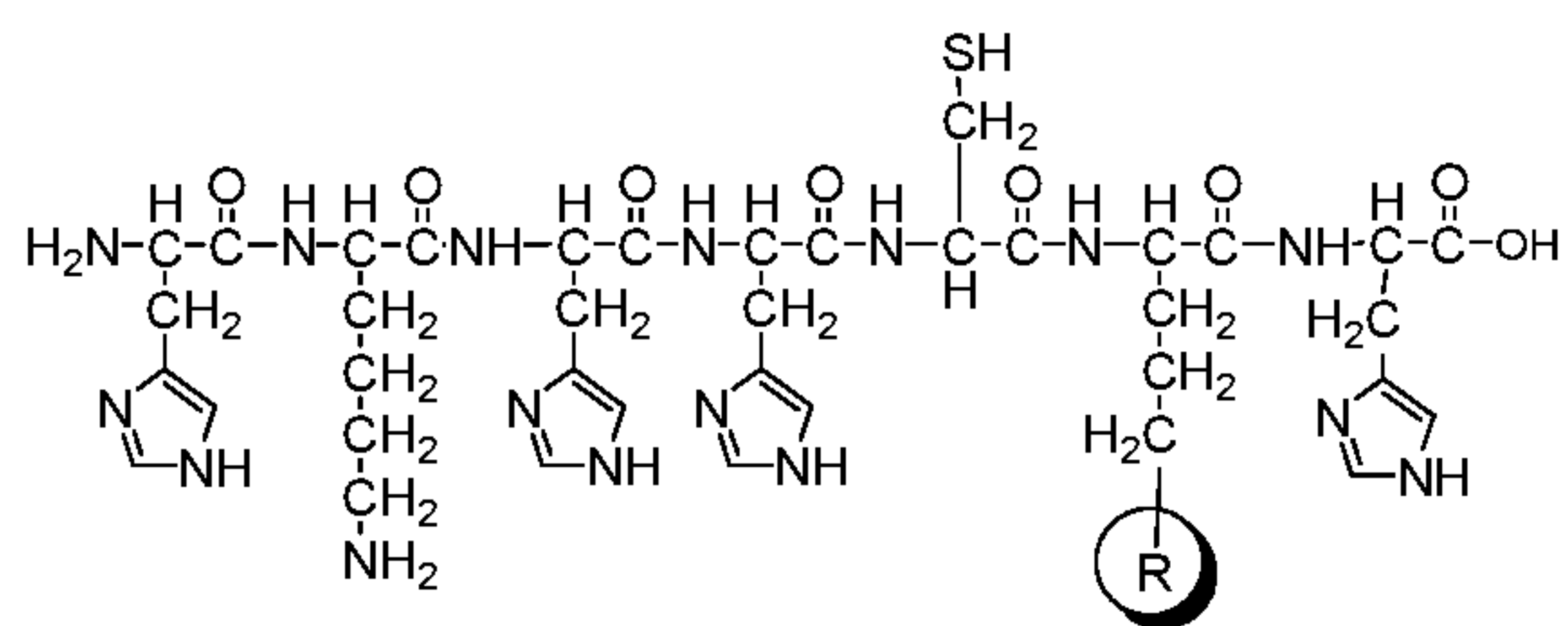
.

An example of a control peptide,

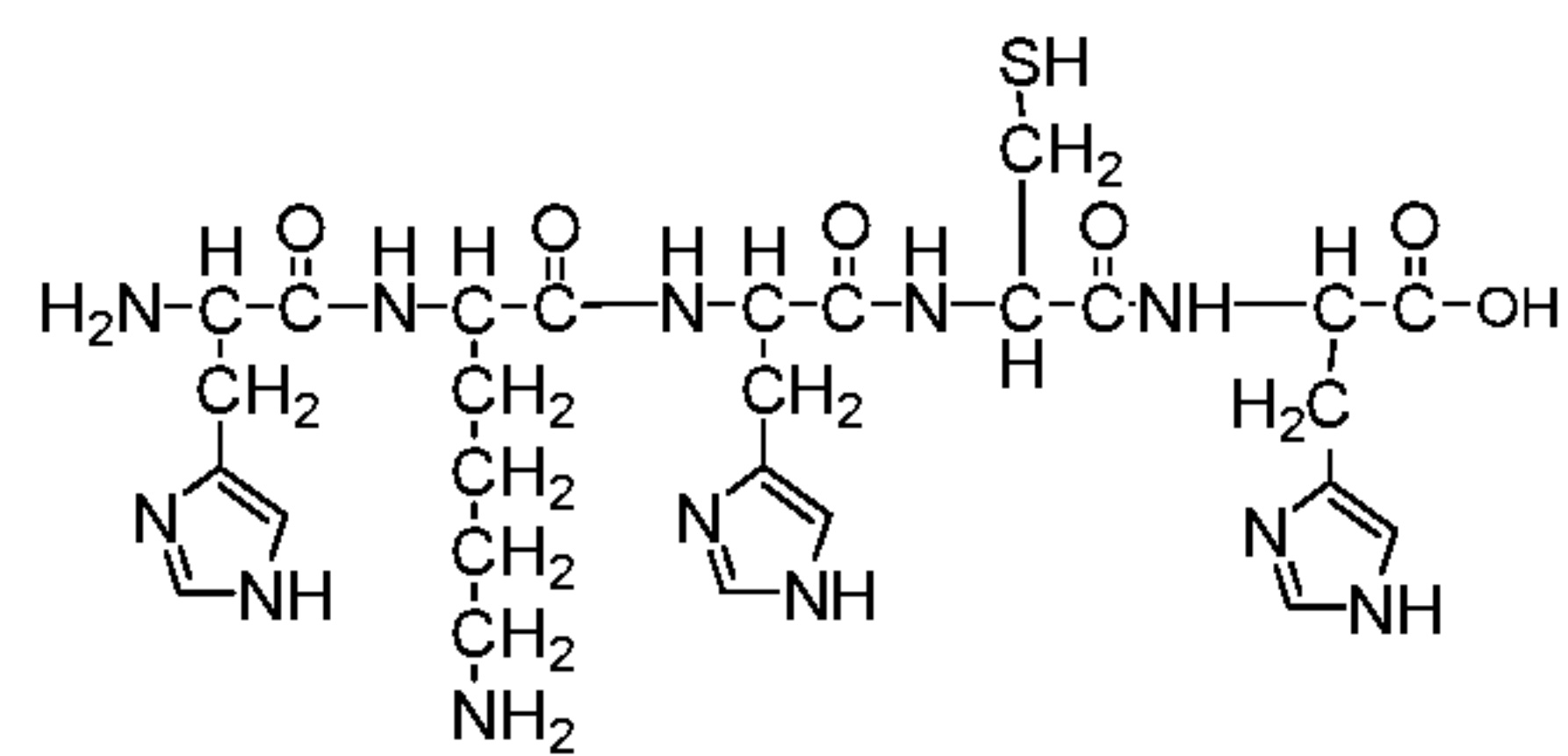


Where the peptide sequence is shorter:

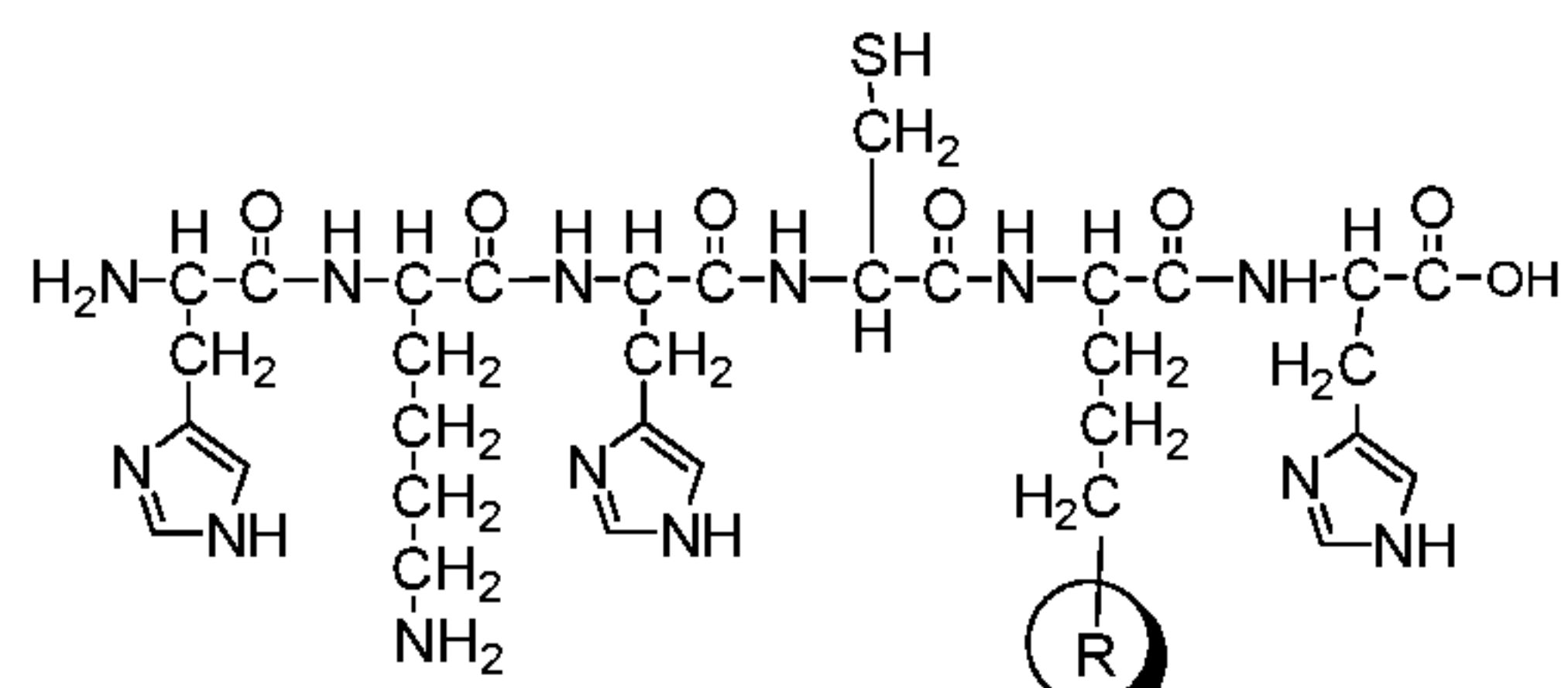
5/16



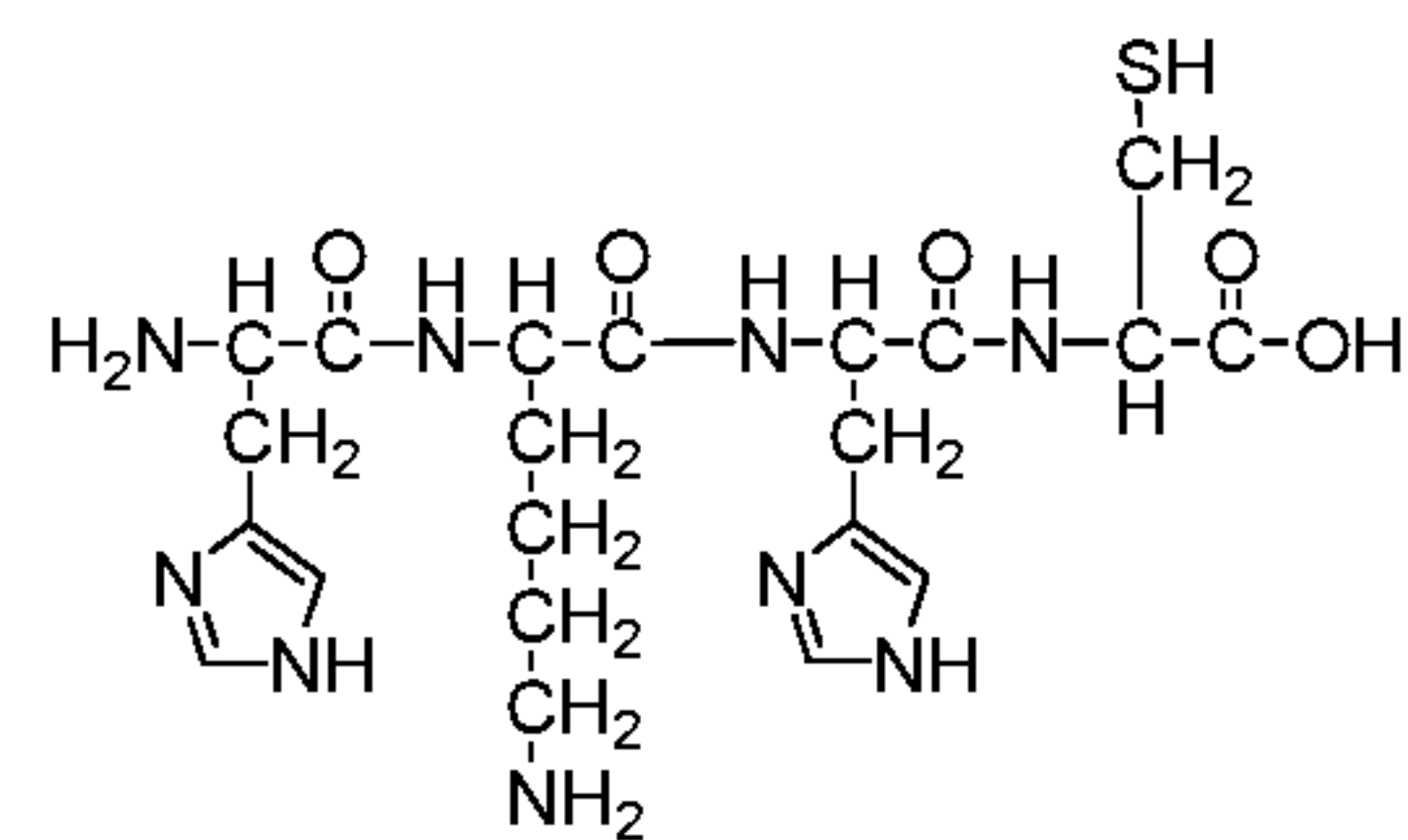
PDoV 3



PDoV 5



PDoV 4

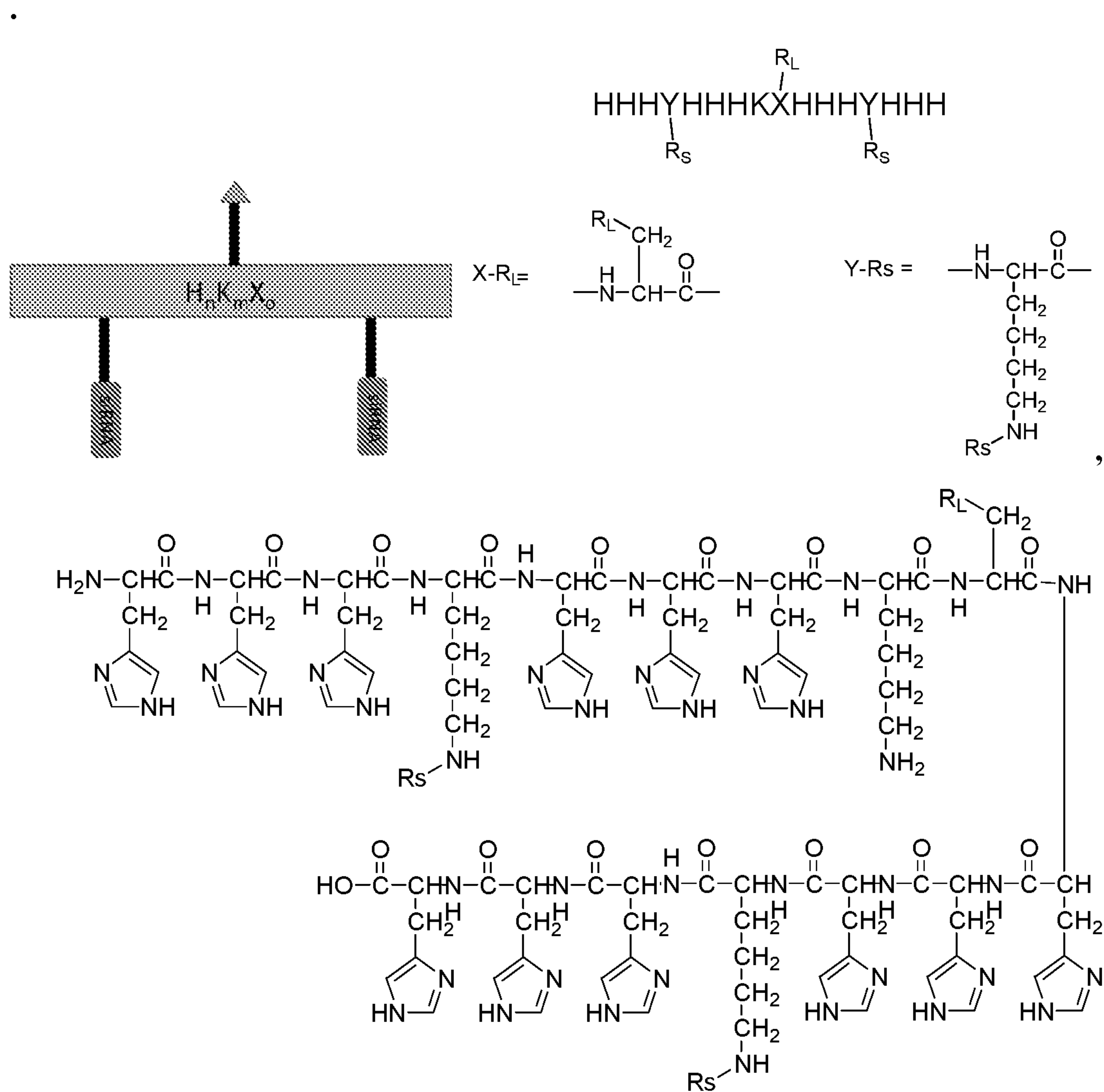


PDoV 6

R = CH_2NH_2
R = $\text{NHC}(\text{NH}_2)_2$

6/16

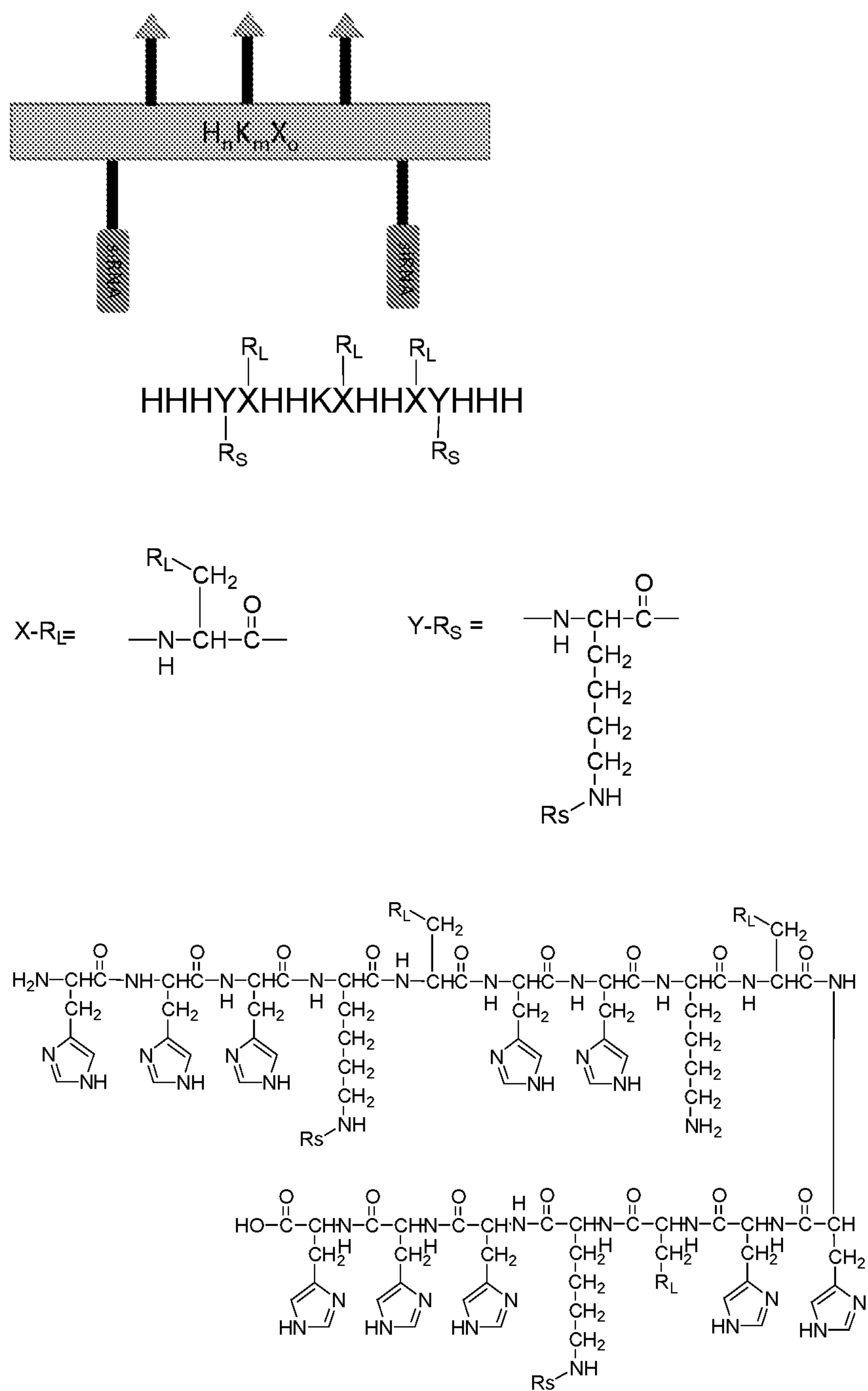
Figure 7.



$X-R_L$ = ligand conjugating site, such as SH, N_3 , maleimide etc.

$X-R_S$ = siRNA, oligonucleotide conjugation site.

Figure 8.



X-R_L = ligand conjugating site, such as SH, N₃, maleimide etc.

X-R_S = siRNA, oligonucleotide conjugation site.

Figure 9A.

Linkage selection for the conjugation sites.

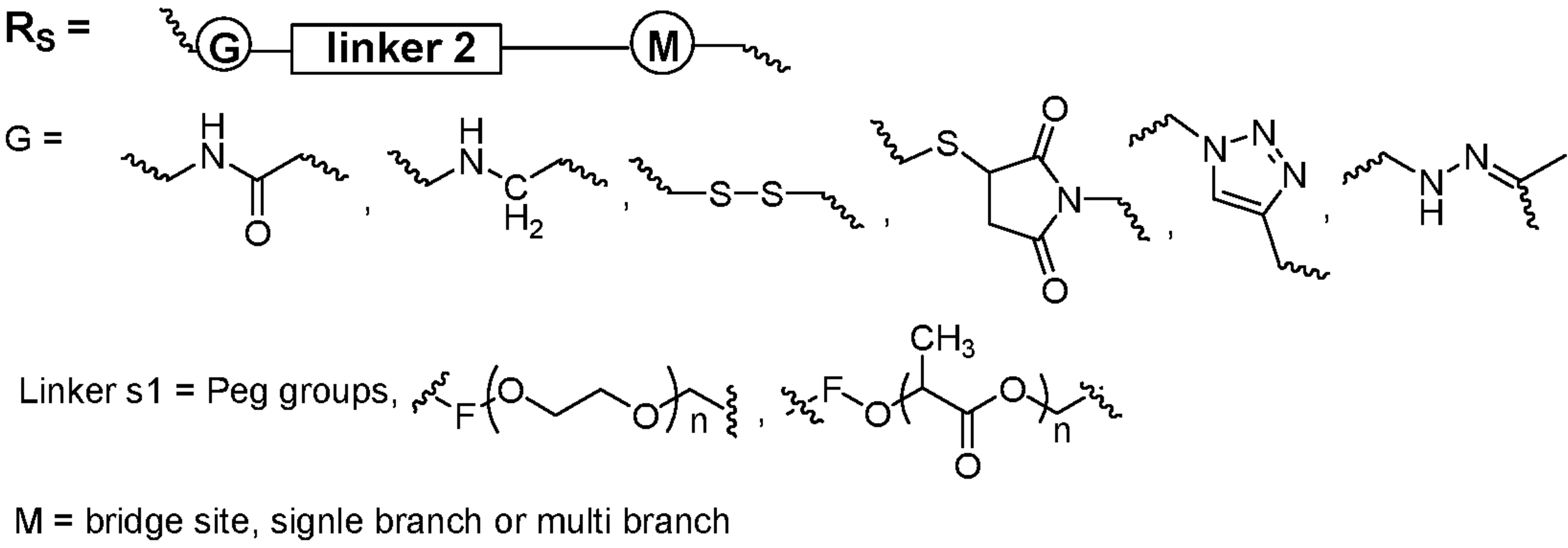


Figure 9B

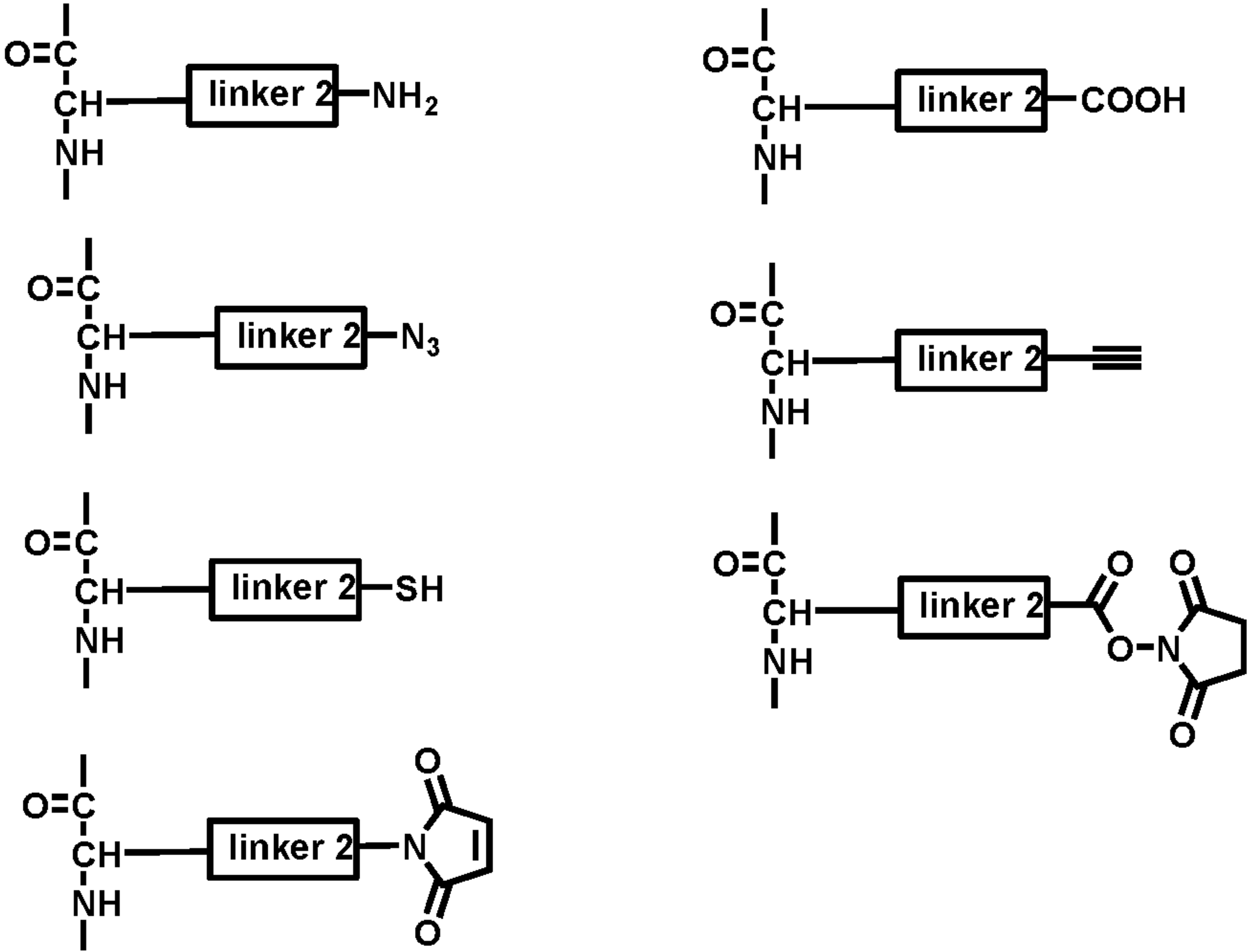
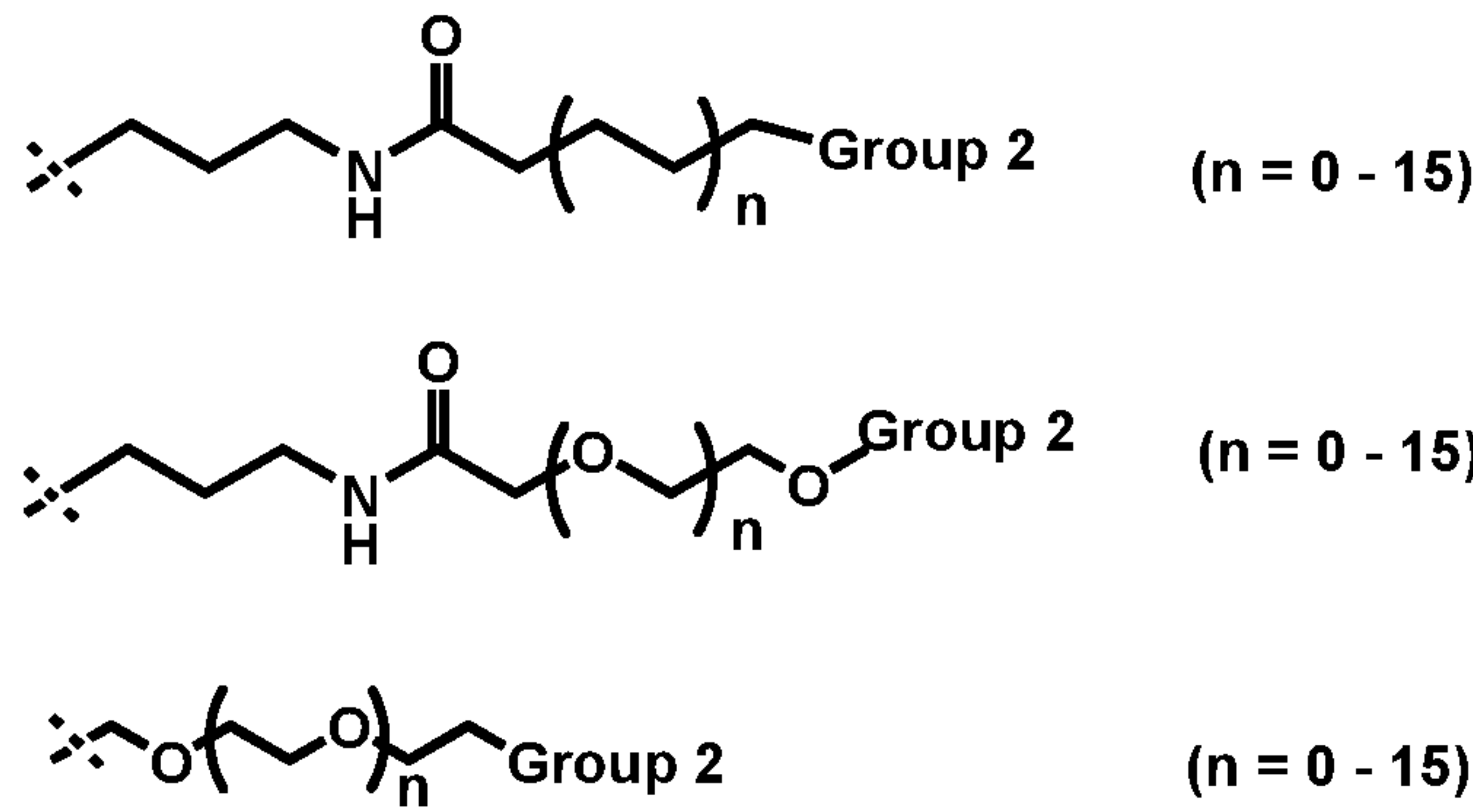
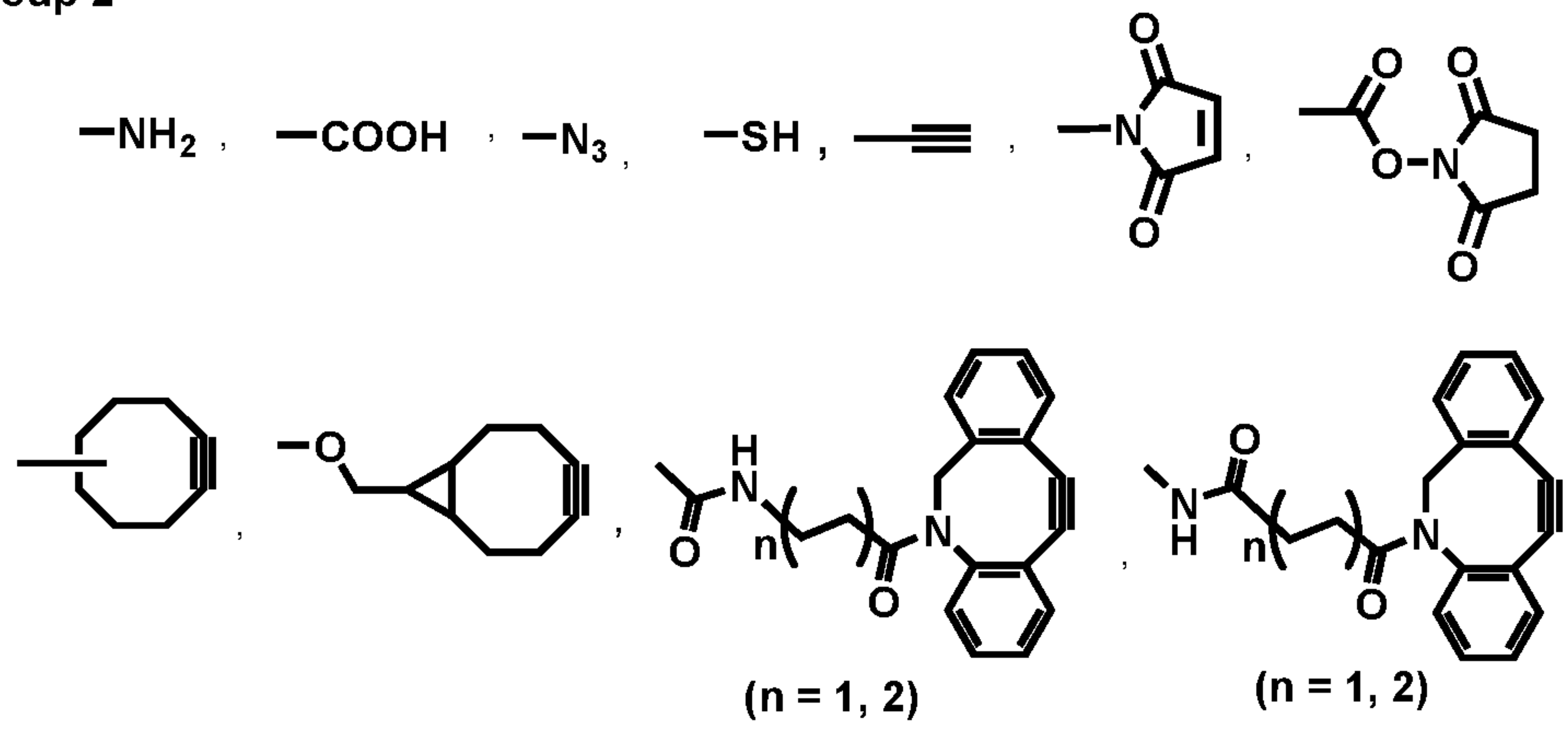


Figure 9C

Linker 2 =



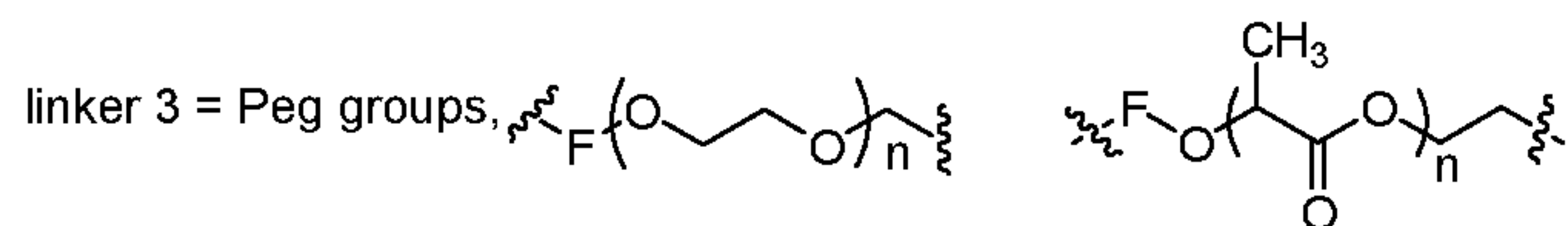
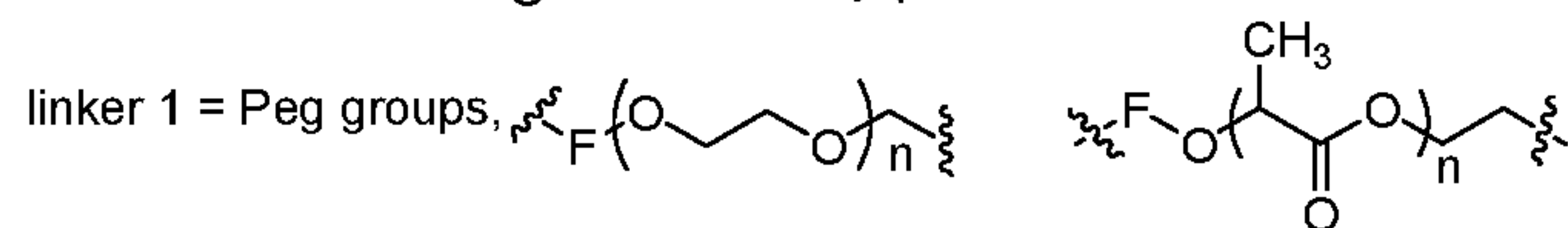
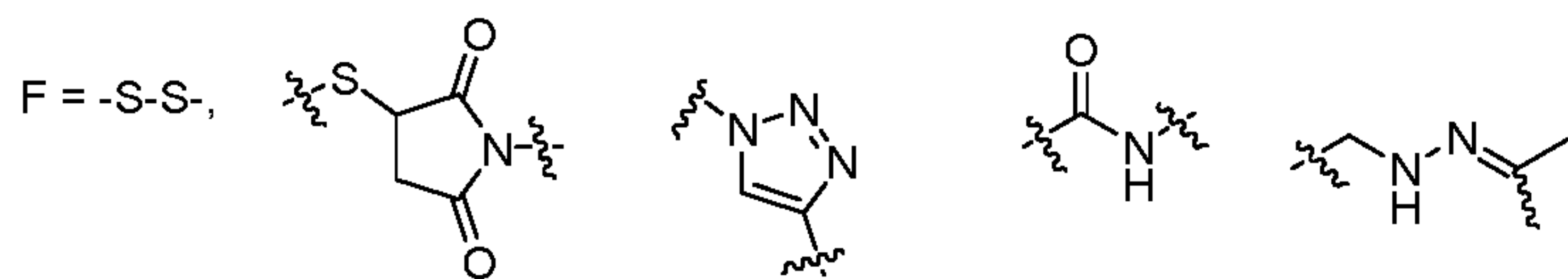
Group 2 =



10/16

Figure 10.**Example of the linkage selection for the ligand conjugation sites.**

L = ligands, GalNAc, peptide, protein



M = bridge site, single branch or multi branch

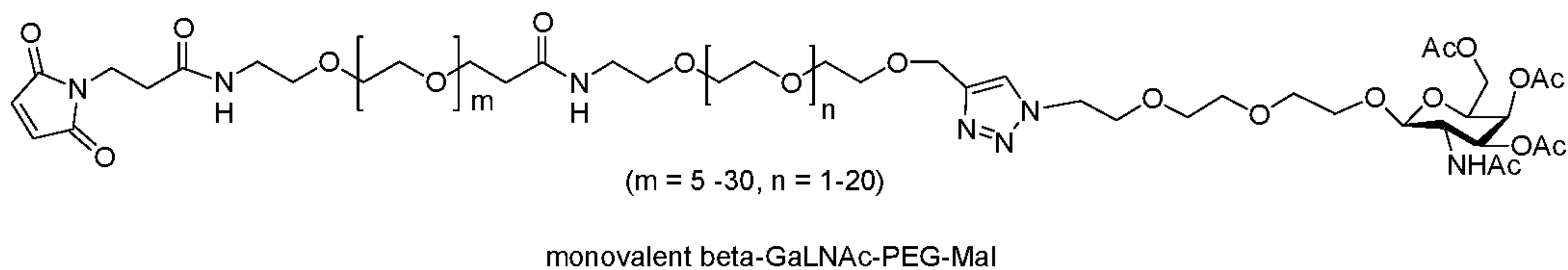
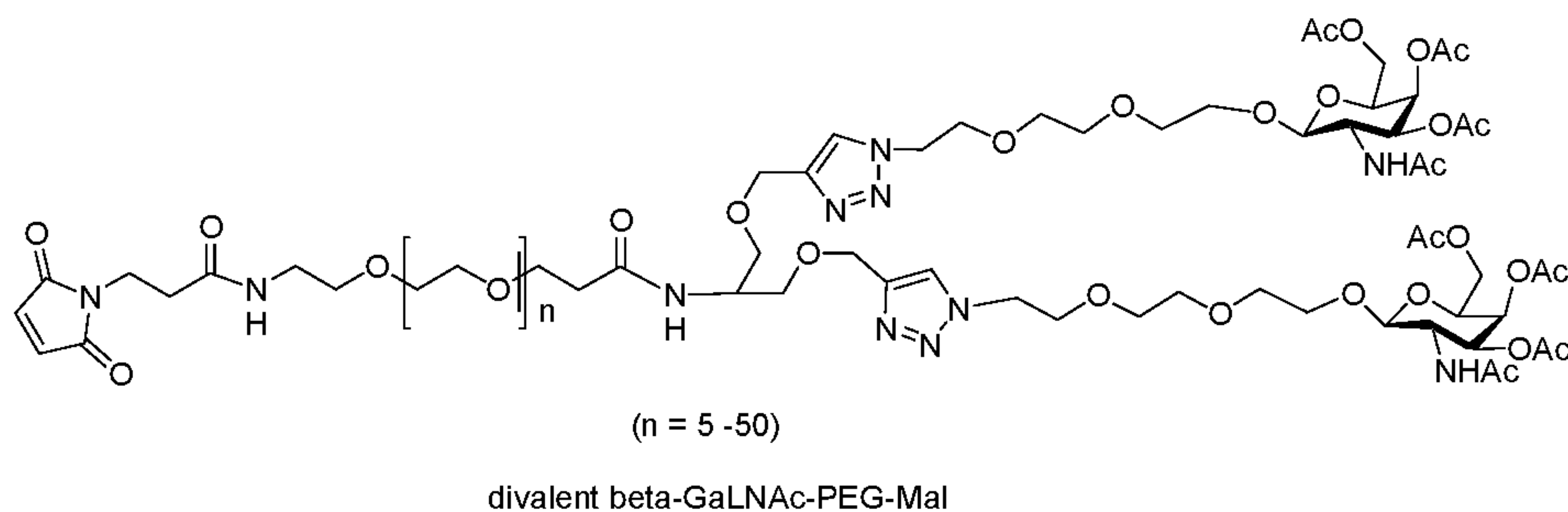
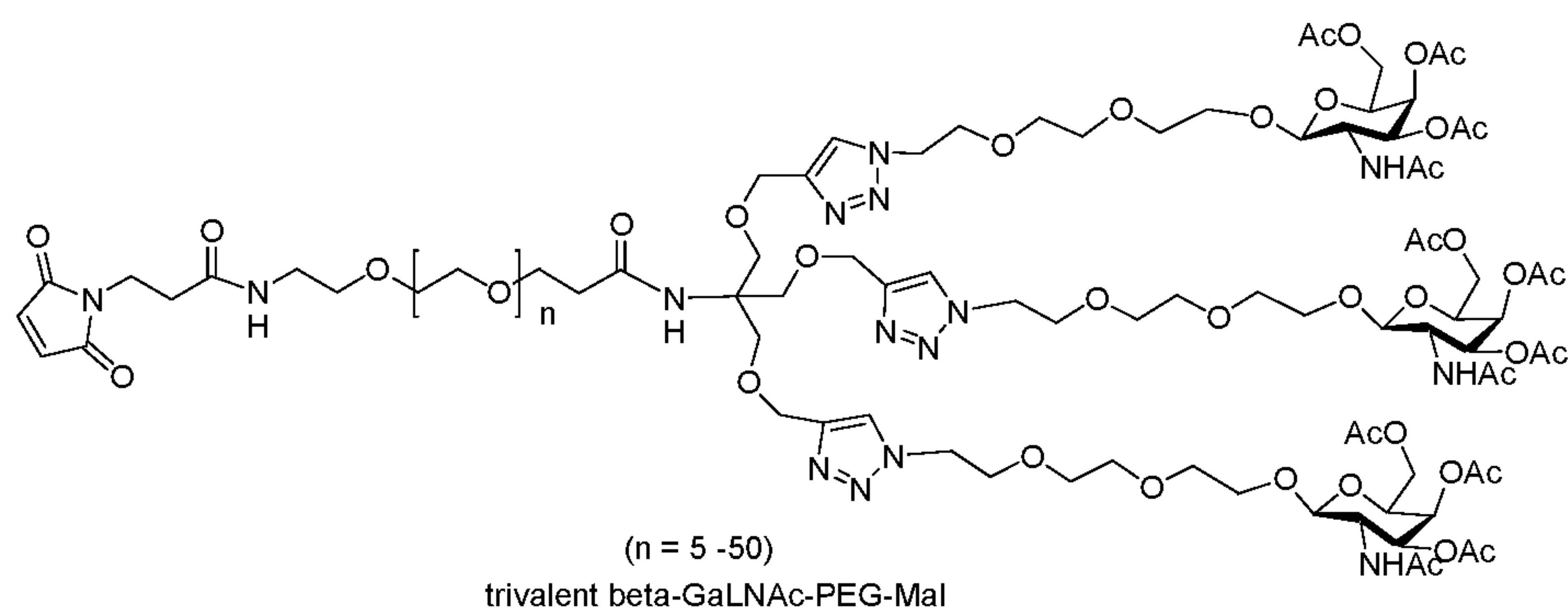
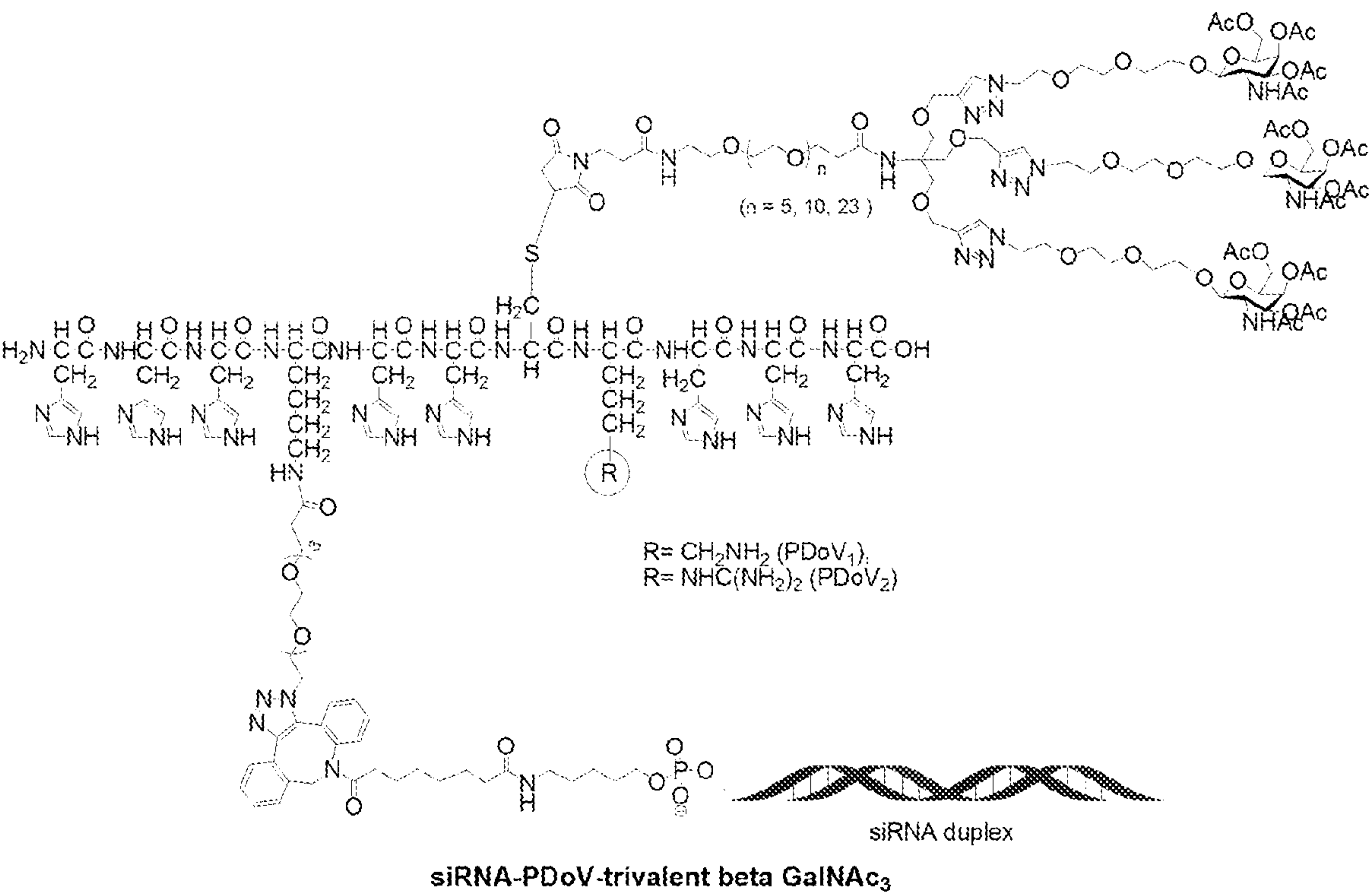


Figure 12.
Representative example of construction of the siRNA- PDoV-ligand compound 2.



13/16

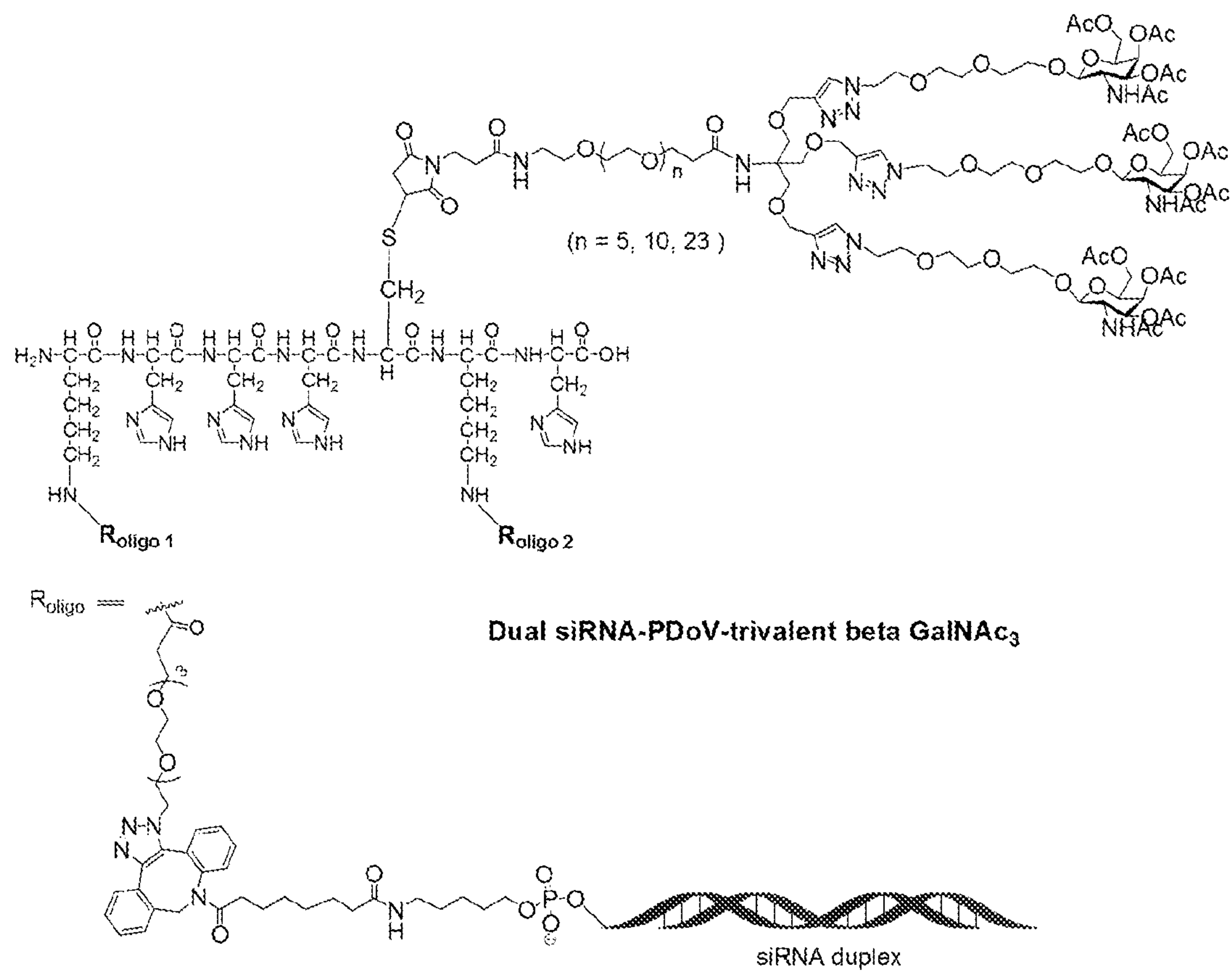
Figure 13.**Example of construction of the dual siRNA- PDoV-ligand compound 3.**

Figure 14.
siRNA sequence and structure modifications.

Sequence	Sequence and modifications
ApoB100a sense	5'-DBCO-C6NHS)(NHC6)GGAAUCuuAuAuuuGAUCcAA-3' (5' end DBCO modification)
ApoB100a Antisense	5'-uuGGAUcAAAuAuAAGAuUCccu-3'
ApoB100b sense	S: 5'-DBCO-C6NHS)(NHC6)GGAAUCuuAuAuuuGAUCcAA-3' (5' end DBCO modification)
ApoB100b Antisense	AS: 5'-uuGGAUcAAAuAuAAGAuUCccu-(Fluor 647 fluoro)-3'
Fully modified ApoB100c sense:	Sense 5'-(DBCO-C6NHS)(NHC6)ggaaucUfuAfUfAfuugaucacaa-3'
ApoB100c Antisense-Dye	Antisense 5'-uUfggaUfcaauauAfaGfauucccu-3'
mTTR1 Sense:	5'-(DBCO-C6NHS)(NHC6)AfaCfaGfuGfuUfcUfuGfcUfcUfaUfaAf-3'
mTTR1 antisense	5'-uUfaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu-3'
mTTR2 Sense:	5'-(DBCO-C6NHS)(NHC6)AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAf-3'
mTTR2 antisense	5'-usUfsaUfaGfaGfcAfaGfaAfcAfcUfgUfusUfsu-3'
25 NSC control Sense:	5'- DBCO-C6NHS)(NHC6)cGfagcaggguaucGfacgauuacaaa-3'
25 NSC control Antisense:	5'-uuuGfTaaucgucgauAfcccugcucg-3'
21 NSC control Sense:	Sense -5'-(DBCO-C6NHS)(NHC6)caggguaFuCfGfAfcgauuacaaa-3'
21 NSC control Antisense:	Antisense 5'- uUfuguAfaucgucgAfuAfcccug-3'
Notes	2'-modification in all sequence: Uppercase letters indicate 2'-OH; lower case letters indicate 2'-O-methyl(2'-OMe) modification, upper case X= normal nucleotide, Xf=2'-F modification.

Figure 15A

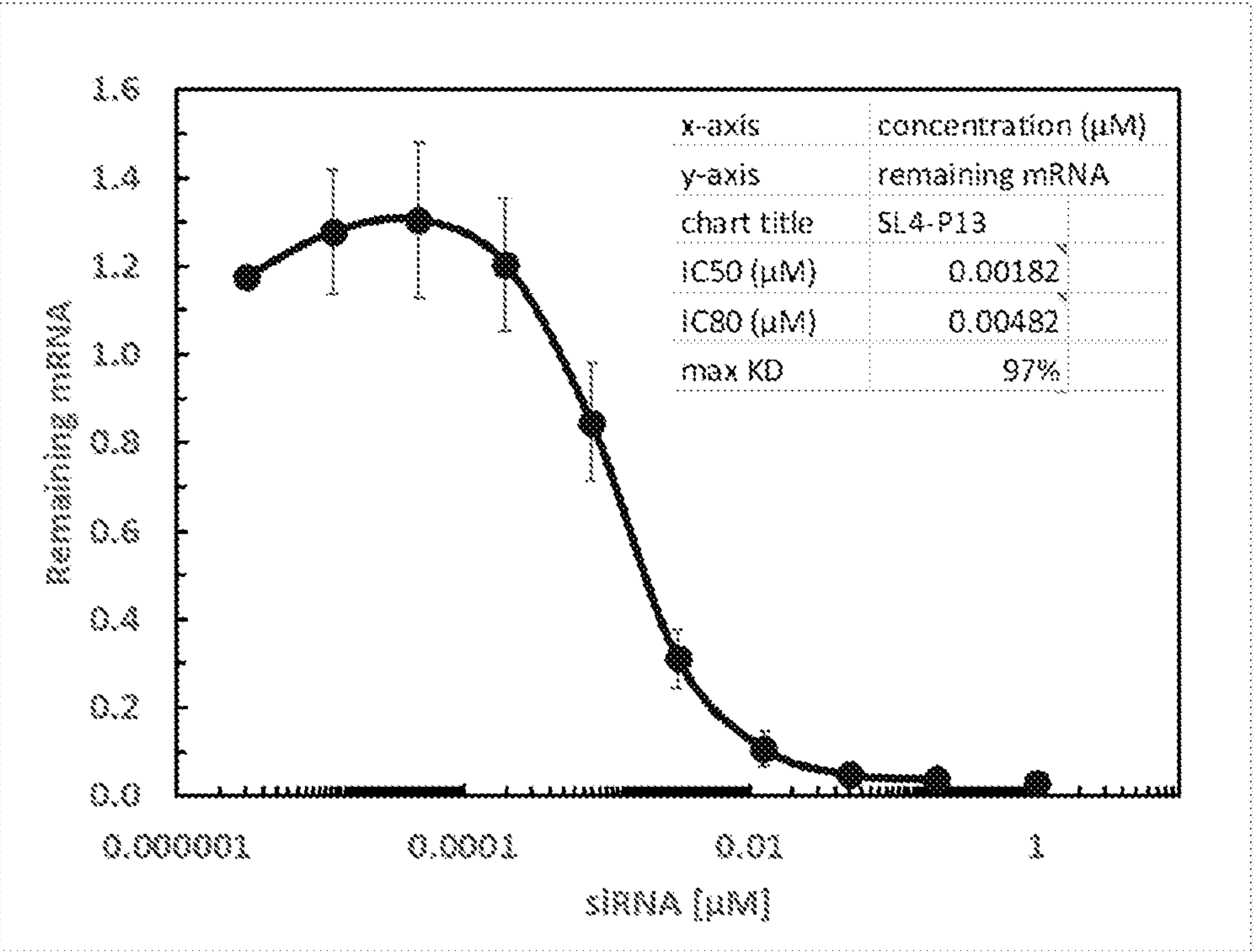


Figure 15B

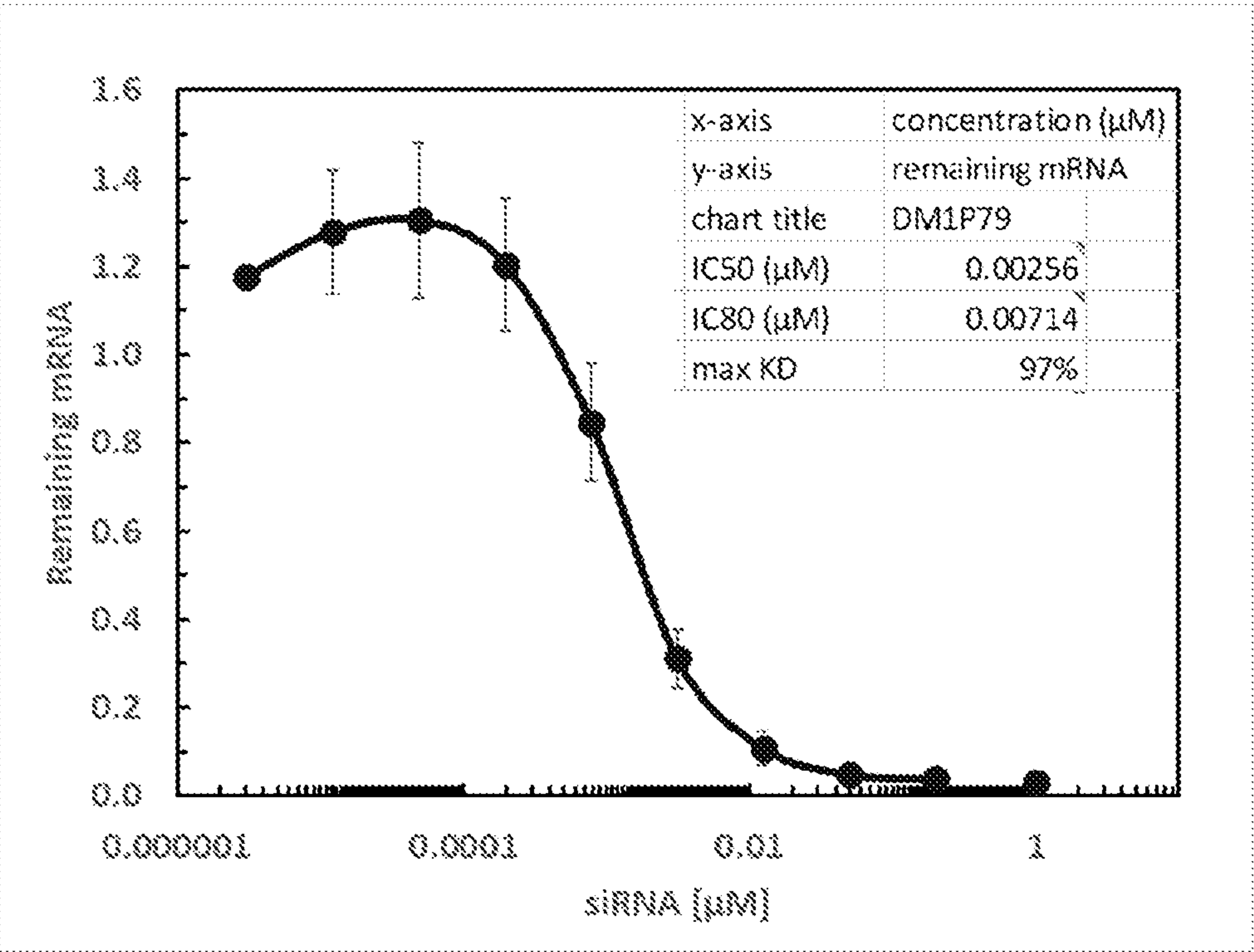


Figure 15C:

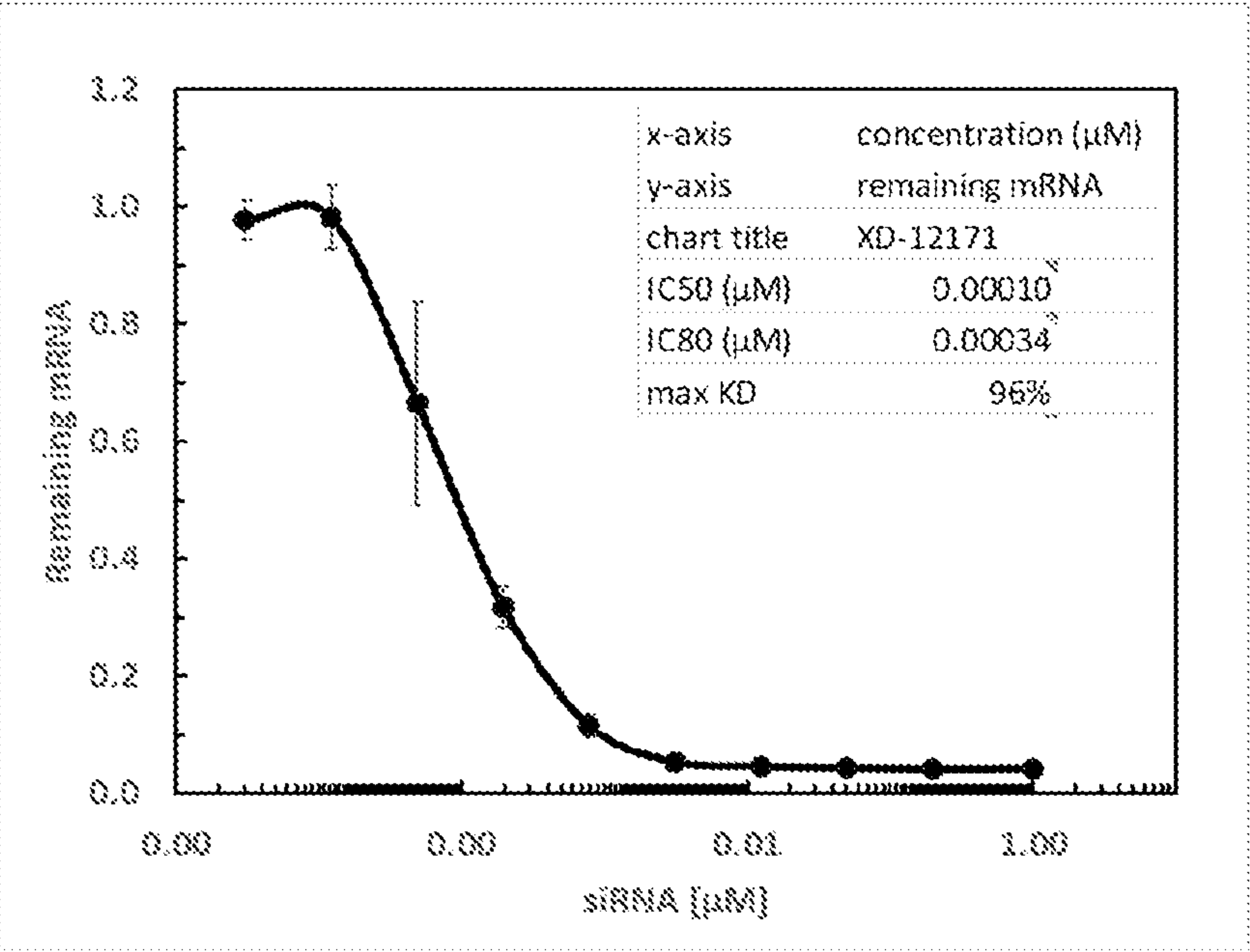


Figure 15D:

