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(54) **Title:** METHODS FOR TREATING HEPATITIS B INFECTIONS

(57) **Abstract:** Certain embodiments of the invention provide a method for identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor, such a method comprising detecting a hepatitis B virus (HBV) infected patient's genotype at one or more of the IL28B/A associated SNPs described herein, wherein the relevant genotype(s) described herein are indicative of a patient that has a higher likelihood of responding to an HBV antigen inhibitor as compared to an HBV infected patient having different genotypes at these locations.

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# METHODS FOR TREATING HEPATITIS B INFECTIONS

## CROSS-REFERENCE TO RELATED APPLICATION

This patent application claims the benefit of priority of U.S. Application Serial No. 62/556,870 filed September 11, 2017, which application is herein incorporated by reference.

## BACKGROUND

Hepatitis B virus (abbreviated as "HBV") is a member of the Hepadnavirus family. The virus particle (sometimes referred to as a virion) includes an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity. The outer envelope contains embedded proteins that are involved in viral binding of, and entry into, susceptible cells, typically liver hepatocytes. In addition to the infectious viral particles, filamentous and spherical bodies lacking a core can be found in the serum of infected individuals. These particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus.

The genome of HBV is made of circular DNA, but it is unusual because the DNA is not fully double-stranded. One end of the full length strand is linked to the viral DNA polymerase. The genome is 3020–3320 nucleotides long (for the full-length strand) and 1700–2800 nucleotides long (for the shorter strand). The negative-sense (non-coding) is complementary to the viral mRNA. The viral DNA is found in the nucleus soon after infection of the cell. There are four known genes encoded by the genome, called C, X, P, and S. The core protein is coded for by gene C (HBcAg), and its start codon is preceded by an upstream in-frame AUG start codon from which the pre-core protein is produced. HBeAg is produced by proteolytic processing of the pre-core protein. The DNA polymerase is encoded by gene P. Gene S is the gene that codes for the surface antigen (HBsAg). The HBsAg gene is one long open reading frame but contains three in frame "start" (ATG) codons that divide the gene into three sections, pre-S1, pre-S2, and S. Because of the multiple start codons, polypeptides of three different sizes called large, middle, and small are produced. The function of the protein coded for by gene X is not fully understood but it is associated with the development of liver cancer. Replication of HBV is a complex process. Although replication takes place in the liver, the virus spreads to the blood where viral proteins and antibodies against them are found in infected people. The

structure, replication and biology of HBV is reviewed in D. Glebe and C.M.Bremer, Seminars in Liver Disease, Vol. 33, No. 2, pages 103-112 (2013).

Infection of humans with HBV can cause an infectious inflammatory illness of the liver. Infected individuals may not exhibit symptoms for many years. It is estimated that about a third of the world population has been infected at one point in their lives, including 350 million who are chronic carriers. The virus is transmitted by exposure to infectious blood or body fluids. Perinatal infection can also be a major route of infection. The acute illness causes liver inflammation, vomiting, jaundice, and possibly death. Chronic hepatitis B may eventually cause cirrhosis and liver cancer.

Although most people who are infected with HBV clear the infection through the action of their immune system, some infected people suffer an aggressive course of infection (fulminant hepatitis); while others are chronically infected thereby increasing their chance of liver disease. Several medications are currently approved for treatment of HBV infection, but infected individuals respond with various degrees of success to these medications. Over the past few decades, several host and viral factors have been found to be associated with differences in HBV clearance or persistence. However, an unexplained variability in treatment outcome still exists, suggesting that the genetic background of the host plays an important role.

Thus, there is a need to identify new biomarkers that can be used to assess the likelihood that an HBV infected patient will respond positively to a particular treatment regimen.

## BRIEF SUMMARY

Accordingly, certain embodiments of the invention provide a method of identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor comprising detecting the hepatitis B virus (HBV) infected patient's genotype at rs12079860, wherein a C/C genotype at rs12079860 is indicative of a patient that has a higher likelihood of responding to an HBV antigen inhibitor as compared to an HBV infected patient having a different genotype at rs12079860.

Certain embodiments of the invention provide a method of identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor comprising:

a) analyzing a biological sample obtained from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860; and

b) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) analyzing a biological sample obtained from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860; and

b) administering an effective amount of an HBV antigen inhibitor to a patient having a C/C genotype at rs12079860.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) analyzing a biological sample obtained from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860;

b) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

c) administering an effective amount of an HBV antigen inhibitor to the patient.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) analyzing a biological sample from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860;

b) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

c) administering an effective amount of an HBV antigen inhibitor to the patient, wherein the HBV inhibitor comprises siRNA 67m (SEQ ID NO:142 and 143), siRNA 71m (SEQ ID NO:144 and 145) and siRNA 74m (SEQ ID NO:10 and 24).

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) obtaining a biological sample from the hepatitis B virus (HBV) infected patient;

b) analyzing the sample to detect the patient's genotype at rs12079860;

c) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

d) administering an effective amount of an HBV antigen inhibitor to the patient, wherein the HBV inhibitor comprises siRNA 67m (SEQ ID NO:142 and 143), siRNA 71m (SEQ ID NO:144 and 145) and siRNA 74m (SEQ ID NO:10 and 24).

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient, comprising administering to the patient an effective amount of an HBV antigen inhibitor, wherein the patient had been determined to have a C/C genotype at rs12079860.

Certain embodiments of the invention provide an HBV antigen inhibitor for the prophylactic or therapeutic treatment of a hepatitis B virus infection in a patient determined to have a C/C genotype at rs12079860.

Certain embodiments of the invention provide the use of an HBV antigen inhibitor to prepare a medicament for treating a hepatitis B virus infection in a patient determined to have a C/C genotype at rs12079860.

Certain embodiments of the invention provide a kit comprising:

a) an HBV antigen inhibitor;

b) instructions for administering the inhibitor to a hepatitis B virus (HBV) infected patient determined to have a C/C genotype at rs12079860.

Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and any figures.

## BRIEF DESCRIPTION OF THE FIGURES

**Figure 1**: Illustrates an intermediate compound of formula Ie, wherein a targeting ligand/linker is bound to a solid phase support, and wherein $Pg^1$ is the protecting group DMTr.

**Figure 2**: Illustrates a representative compound of formula Id wherein a targeting ligand is bound to a solid phase support, with a nucleic acid covalently bound.

**Figure 3**: Illustrates a representative compound of formula Id, wherein a targeting ligand-nucleic acid conjugate has been cleaved from a solid phase support and deprotected to provide the compound of formula I.

## DETAILED DESCRIPTION

As described herein, a series of single nucleotide polymorphisms (SNPs) have been identified as biomarkers that may be used to detect patients infected with HBV that have a higher likelihood of responding to an HBV antigen inhibitor. These SNPs are located near

the *interleukin 28B* (*IL-28B*) and the *interleukin 28A (IL-28A)* genes on chromosome 19 (National Center for Biotechnology Information, Entrez Gene Entry for IL28B, Gene ID: 282617; Entrez Gene Entry for IL28A, Gene ID: 282616). *IL28B* encodes IFN-λ3, which induces antiviral activity by itself and through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) complex, which induces IFN-stimulated genes (ISGs). *IL28A* encodes IFN-λ2. In certain embodiments, there is a correlation between such SNPs associated with these genes and with increased IFN-λ2 and/or IFN-λ3 expression. The table shown below includes a non-limiting list of these *IL28A/B* SNPs, which may be used in methods of the invention described herein.

| IL28B/A associated SNPs | Relevant Genotype |
|---|---|
| rs12979860 | CC (CT*) |
| rs8099917 | TT (TG*) |
| rs12980275+ | AA |
| rs8105790 | CC, TC |
| rs11881222 | TT, AG |
| rs8103142+ | AA, TC |
| rs28416813 | CG |
| rs4803219 | CT |
| rs7248668 | GA |
| rs368234815+,^ | TT |
| rs8109886 | AA |

* combination of: rs12979860CT/rs8099917TG; or rs1297980CT/rs809917TT. + indicates that these SNPs have been reported to be in high linkage disequilibrium with rs12979860. ^ rs368234815 is located upstream of IFNL3/IL28B; a frameshift variant gives rise to IFNL4. *See, e.g.,* Tanaka et al., Nat Genet 2009, 41:1105-1109; Fischer et al., Hepatol. 2012, 55(6):1700-10; Aziz et al., Int J Infect Dis 2015, 30:91-7; Shaikh et al., J Med Virol, 2015, 87(5):814-20; Karatayli et al., Liver Int. 2015, 35(3):846-53; Juniastuti et al., J Clin Microbiol 2014, 52(6): 2193–2195; Prokunina-Olsson et al., Nat Gen 2013, 45(2):164-71.

Accordingly, certain embodiments of the invention provide a method of identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor comprising detecting the hepatitis B virus (HBV) infected patient's genotype at any one or a combination of the SNPs listed in the table above, wherein a patient having the relevant genotype or combination of genotypes has a higher likelihood of responding to an HBV antigen inhibitor as compared to an HBV infected patient having different genotypes at these locations. In certain

embodiments, at least one SNP from the above table is detected. In certain embodiments, a combination of at least 2 SNPs from the above table are detected. In certain embodiments, a combination of at least 3 SNPs from the above table are detected. In certain embodiments, a combination of at least 4 SNPs from the above table are detected. In certain embodiments, a combination of at least 5 SNPs from the above table are detected.

In certain embodiments of the invention, a patient's genotype at the rs12979860 SNP is detected. The rs12979860 SNP is located approximately 3 kb upstream of the *IL-28B* gene (*see, e.g.*, Ge et al., Nature 2009, 461:399-401). As described in the Examples, it has been advantageously shown that individuals having a C/C genotype at rs12979860 have a higher likelihood of positively responding to an HBV antigen inhibitor treatment than individuals having another genotype at this location. Additionally, patients having a combination of a CT genotype at rs12979860 and a TG or TT genotype at rs8099917 may also have a higher likelihood of positively responding to an HBV antigen inhibitor treatment.

In certain embodiments of the invention, a patient's genotype at the rs8099917 SNP is detected. The rs8099917 SNP is located in an intergenic region between *IL28A* and *IL28B*, ~8kb downstream from *IL28B* and ~16kb upstream from *IL28A* (*see, e.g.,* Suppiah et al., Nat Genet 2009, 41:1100-1104; Tanaka et al., Nat Genet 2009, 41:1105-1109). In certain embodiments, a T/T genotype at rs8099917 is indicative of a HBV infected patient that has a higher likelihood of responding to an HBV antigen inhibitor, as compared to an HBV infected patient having a different genotype at this location. Additionally, in certain embodiments, a patient having combination of a T/G genotype at rs8099917 and C/T genotype at rs12979860 may also have a higher likelihood of positively responding to an HBV antigen inhibitor treatment.

In certain embodiments of the invention, a patient's genotype at the rs12980275 SNP is detected. rs12980275 is also located near the *IL28B* gene (*see, e.g.,* Tanaka et al., Nat Genet 2009, 41:1105-1109). In certain embodiments, an A/A genotype at rs12980275 is indicative of a HBV infected patient that has a higher likelihood of responding to an HBV antigen inhibitor, as compared to an HBV infected patient having a different genotype at this location.

Thus, certain embodiments of the invention provide a method of identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor comprising detecting the hepatitis B virus (HBV) infected patient's genotype at rs12079860, rs8099917 and/or rs12980275, wherein a C/X genotype at rs12079860, a T/Z genotype at rs8099917 and/or an A/A genotype at rs12980275 is indicative of a patient that has a higher likelihood of responding to an HBV antigen inhibitor as compared to an HBV infected patient having different genotypes

at rs12079860, rs8099917 and/or rs12980275, wherein X is C or T; and Z is T or G. In certain embodiments, X is C. In certain embodiments, Z is T. In certain embodiments, X is T and Z is G. In certain embodiments, X is T and Z is T. In certain embodiments, the hepatitis B virus (HBV) infected patient's genotype at rs12079860 is detected. In certain embodiments, the hepatitis B virus (HBV) infected patient's genotype at rs8099917 is detected. In certain embodiments, the hepatitis B virus (HBV) infected patient's genotype at rs12980275 is detected.

Certain embodiments of the invention provide a method of identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor comprising detecting the hepatitis B virus (HBV) infected patient's genotype at rs12079860, wherein a C/C genotype at rs12079860 is indicative of a patient that has a higher likelihood of responding to an HBV antigen inhibitor as compared to an HBV infected patient having a different genotype at rs12079860.

Certain embodiments of the invention provide a method of identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor comprising:

a) analyzing a biological sample obtained from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860; and

b) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860.

In certain embodiments of the invention, a method described herein further comprises obtaining a biological sample from the hepatitis B virus (HBV) infected patient.

Thus, certain embodiments of the invention provide a method of identifying a patient that has a higher likelihood of responding to an HBV antigen inhibitor comprising:

a) obtaining a biological sample from the hepatitis B virus (HBV) infected patient; and

b) analyzing the sample to detect the patient's genotype at rs12079860; and

c) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860.

In certain embodiments, a method described herein further comprises administering an effective amount of an HBV antigen inhibitor to the HBV infected patient having a C/C genotype at rs12079860.

In certain embodiments, an effective amount of an HBV antigen inhibitor for a patient having a C/C genotype at rs12079860 is less than an effective amount of an HBV antigen inhibitor for a patient having a different genotype at rs12079860. Thus, in certain embodiments,

an HBV infected patient having a C/C genotype at rs12079860 is administered a different HBV antigen inhibitor treatment regimen than an HBV infected patient having a different genotype at rs12079860. In certain embodiments, the HBV infected patient having a C/C genotype at rs12079860 is administered a lower dosage of the HBV antigen inhibitor and/or is administered the HBV antigen inhibitor for a shorter period of time as compared to an HBV infected patient having a different genotype at rs12079860.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) analyzing a biological sample obtained from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860; and

b) administering an effective amount of an HBV antigen inhibitor to a patient having a C/C genotype at rs12079860.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) obtaining a biological sample from the hepatitis B virus (HBV) infected patient;

b) analyzing the sample to detect the patient's genotype at rs12079860; and

c) administering an effective amount of an HBV antigen inhibitor to a patient having a C/C genotype at rs12079860.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) analyzing a biological sample obtained from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860;

b) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

c) administering an effective amount of an HBV antigen inhibitor to the patient.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) obtaining a biological sample from the hepatitis B virus (HBV) infected patient;

b) analyzing the sample to detect the patient's genotype at rs12079860;

c) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

d) administering an effective amount of an HBV antigen inhibitor to the patient.

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) analyzing a biological sample from the hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860;

b) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

c) administering an effective amount of an HBV antigen inhibitor to the patient, wherein the HBV inhibitor comprises siRNA 67m (SEQ ID NO:142 and 143), siRNA 71m (SEQ ID NO:144 and 145) and siRNA 74m (SEQ ID NO:10 and 24).

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient comprising:

a) obtaining a biological sample from the hepatitis B virus (HBV) infected patient;

b) analyzing the sample to detect the patient's genotype at rs12079860;

c) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

d) administering an effective amount of an HBV antigen inhibitor to the patient, wherein the HBV inhibitor comprises siRNA 67m (SEQ ID NO:142 and 143), siRNA 71m (SEQ ID NO:144 and 145) and siRNA 74m (SEQ ID NO:10 and 24).

Certain embodiments of the invention provide a method of treating a hepatitis B virus (HBV) infected patient, comprising administering to the patient an effective amount of an HBV antigen inhibitor, wherein the patient had been determined to have a C/C genotype at rs12079860.

HBV antigen inhibitors are described in detail below. Thus, in certain embodiments, the HBV antigen inhibitor is an agent described herein.

In certain embodiments, the HBV antigen inhibitor is a core antigen inhibitor.

In certain embodiments, the HBV antigen inhibitor is a surface antigen inhibitor.

In certain embodiments, the HBV antigen inhibitor is selected from an oligonucleotide, a small molecule or a polypeptide.

In certain embodiments, the HBV antigen inhibitor is a small molecule.

In certain embodiments, the HBV antigen inhibitor is an oligonucleotide.

In certain embodiments, the oligonucleotide is a siRNA molecule.

In certain embodiments, the HBV antigen inhibitor is an siRNA molecule selected from the siRNA molecules described in Tables A and B.

In certain embodiments, the HBV antigen inhibitor comprises a combination of two or more siRNA molecules selected from the siRNA molecules described in Tables A and B or the Examples.

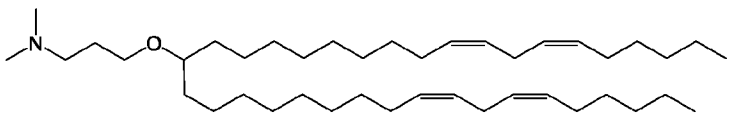
In certain embodiments, the HBV antigen inhibitor comprises a combination of three or more siRNA molecules selected from the siRNA molecules described in Tables A and B or the Examples.

In certain embodiments, the HBV antigen inhibitor comprises siRNA 67m (SEQ ID NO:142 and 143), siRNA 71m (SEQ ID NO:144 and 145) and siRNA 74m (SEQ ID NO:10 and 24).

In certain embodiments, the oligonucleotide (e.g., siRNA) is comprised in a lipid nanoparticle formulation, wherein the lipid nanoparticle formulation comprises a cationic lipid and a non-cationic lipid.

In certain embodiments, the cationic lipid is selected from the group consisting of 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)) , 3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (DLin-MP-DMA; Compound (**508**)), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) (Compound (**507**)), (6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)), a salt thereof, and a mixture thereof.

In certain embodiments, the cationic lipid is:



3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (**508**).

In certain embodiments, the non-cationic lipid is cholesterol or a derivative thereof.

In certain embodiments, the non-cationic lipid is a phospholipid.

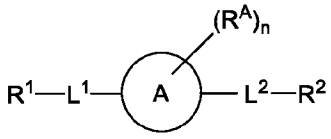
In certain embodiments, the non-cationic lipid is a mixture of a phospholipid and cholesterol or a derivative thereof.

In certain embodiments, the phospholipid is selected from the group consisting of dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and a mixture thereof. In certain embodiments, the phospholipid is DSPC.

In certain embodiments, the lipid formulation further comprises a conjugated lipid that inhibits aggregation of particles. In certain embodiments, the conjugated lipid that inhibits aggregation of particles is a polyethyleneglycol (PEG)-lipid conjugate. In certain embodiments, the PEG-lipid conjugate is selected from the group consisting of a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, a PEG-phospholipid conjugate, a PEG-ceramide (PEG-Cer) conjugate, a PEG- dimyristyloxypropyl (PEG-DMA) conjugate and a mixture thereof. In certain embodiments, the PEG-lipid conjugate is a PEG-C-DMA conjugate.

In certain embodiments, the cationic lipid comprises from about 48 mol % to about 62 mol % of the total lipid present in each particle within the formulation. In certain embodiments, the lipid nanoparticle formulation comprises a phospholipid and cholesterol or cholesterol derivative, wherein the phospholipid comprises from about 7 mol % to about 17 mol % of the total lipid present in each particle within the formulation and the cholesterol or derivative thereof comprises from about 25 mol % to about 40 mol % of the total lipid present in each particle within the formulation. In certain embodiments, the conjugated lipid that inhibits aggregation of particles comprises from about 0.5 mol % to about 3 mol % of the total lipid present in each particle within the formulation.

In certain embodiments, the HBV antigen inhibitor is conjugated to a targeting moiety. In certain embodiments, the HBV antigen inhibitor is an oligonucleotide, such as an siRNA, and the oligonucleotide is conjugated to a targeting moiety. For example, in certain embodiments, the oligonucleotide (e.g., siRNA) is comprised within a compound of formula I




(I)

wherein:

$R^1$ a is targeting ligand;

$L^1$ is absent or a linking group;

$L^2$ is absent or a linking group;

$R^2$ is an oligonucleotide;

the ring A is absent, a 3-20 membered cycloalkyl, a 5-20 membered aryl, a 5-20 membered heteroaryl, or a 3-20 membered heterocycloalkyl;

each $R^A$ is independently selected from the group consisting of hydrogen, hydroxy, CN, F, Cl, Br, I, -$C_{1-2}$ alkyl-$OR^B$, $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl; wherein the $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl are optionally substituted with one or more groups independently selected from halo, hydroxy, and $C_{1-3}$ alkoxy;

$R^B$ is hydrogen, a protecting group, a covalent bond to a solid support, or a bond to a linking group that is bound to a solid support; and

n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

or a salt thereof.

In certain embodiments, methods of the invention further comprise administering at least one additional therapeutic agent. In certain embodiments, the at least one additional therapeutic agent is selected from the group consisting of:

(A) an agent that controls viral replication;

(B) an agent that reduces viral Ags;

(C) an immune enhancer; and

(D) an immune stimulant.

These classes of additional therapeutic agents are further described below as Category I, II and III agents.

In certain embodiments, the HBV infected patient is further infected with hepatitis D virus (HDV).

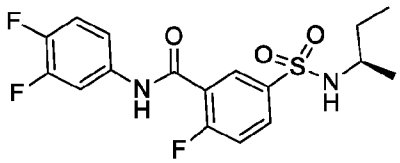
**HBV Antigen Inhibitors**

The term "HBV antigen inhibitor" refers to a compound that can inhibit the expression and/or function of an HBV antigen (*i.e.*, core or surface antigen), either directly or indirectly. The inhibitor may be of natural or synthetic origin. For example, HBV antigen inhibitors, include but are not limited to, e.g., oligonucleotides, small molecules and polypeptides. The term "small molecule" includes organic molecules having a molecular weight of less than about, e.g., 1000 amu. In one embodiment a small molecule can have a molecular weight of less than about 800 amu. In another embodiment a small molecule can have a molecular weight of less than about 500 amu. In certain embodiments, the HBV inhibitor does not comprise interferon (IFN).

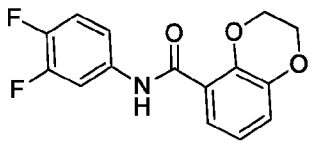
In certain embodiments, the HBV antigen inhibitor is a core antigen inhibitor. As used herein, the term "core antigen inhibitor" refers to a compound that can inhibit the expression and/or function of an HBV core antigen.

For example, in certain embodiments, the HBV core antigen inhibitor is a capsid inhibitor. As described herein the term "capsid inhibitor" includes compounds that are capable of inhibiting the expression and/or function of a capsid protein either directly or indirectly. For example, a capsid inhibitor may include, but is not limited to, any compound that inhibits capsid assembly, induces formation of non-capsid polymers, promotes excess capsid assembly or misdirected capsid assembly, affects capsid stabilization, and/or inhibits encapsidation of RNA. Capsid inhibitors also include any compound that inhibits capsid function in a downstream event(s) within the replication process (e.g., viral DNA synthesis, transport of relaxed circular DNA (rcDNA) into the nucleus, covalently closed circular DNA (cccDNA) formation, virus maturation, budding and/or release, and the like). For example, in certain embodiments, the inhibitor detectably inhibits the expression level or biological activity of the capsid protein as measured, e.g., using an assay described herein. In certain embodiments, the inhibitor inhibits the level of rcDNA and downstream products of viral life cycle by at least 5%, at least 10%, at least 20%, at least 50%, at least 75%, at least 90%, at least 95% or at least 99%.

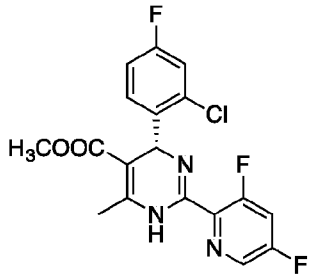
The term capsid inhibitor includes compounds described in International Patent Applications Publication Numbers WO2013006394, WO2014106019, and WO2014089296, including the following compounds:



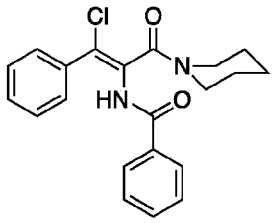

and



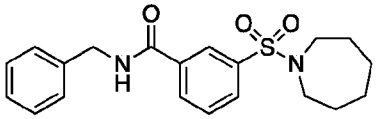

The term capsid inhibitor also includes the compounds **Bay-41-4109** (see International Patent Application Publication Number WO/2013/144129), **AT-61** (see International Patent Application Publication Number WO/1998/33501; and King, RW, et al., Antimicrob Agents Chemother., **1998**, *42*, 12, 3179–3186), **DVR-01** and **DVR-23** (see International Patent Application Publication Number WO 2013/006394; and Campagna, MR, et al., J. of Virology, 2013, 87, 12, 6931, and pharmaceutically acceptable salts thereof:



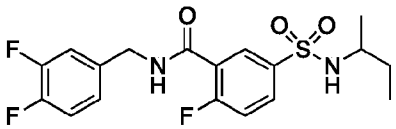

Bay-41-4109




AT-61




DVR-01



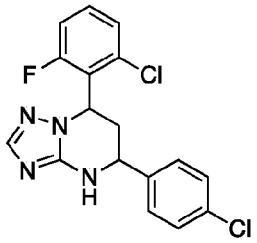

DVR-23

The term capsid inhibitor also includes the compounds Compound 3, GLS-4, and NVR 3-778.

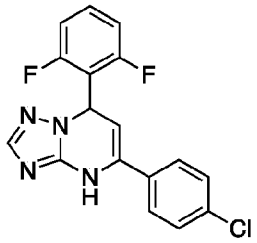
In certain embodiments, the HBV antigen inhibitor is a surface antigen inhibitor (sAg inhibitor). As described herein the term "surface antigen inhibitor" includes compounds that are capable of inhibiting the expression and/or function of a surface antigen either directly or indirectly.

For example, in certain embodiments, the surface antigen inhibitor is a sAg secretion inhibitor. As described herein the term "sAg secretion inhibitor" includes compounds that are capable of inhibiting, either directly or indirectly, the secretion of sAg (S, M and/or L surface antigens) bearing subviral particles and/or DNA containing viral particles from HBV-infected cells. For example, in certain embodiments, the inhibitor detectably inhibits the secretion of sAg as measured, e.g., using assays known in the art or described herein, e.g., ELISA assay or by Western Blot. In certain embodiments, the inhibitor inhibits the secretion of sAg by at least 5%, at least 10%, at least 20%, at least 50%, at least 75%, or at least 90%. In certain embodiments, the inhibitor reduces serum levels of sAg in a patient by at least 5%, at least 10%, at least 20%, at least 50%, at least 75%, or at least 90%.

The term sAg secretion inhibitor includes compounds described in United States Patent Number 8,921,381, as well as compounds described in United States Patent Application Publication Numbers 2015/0087659 and 2013/0303552. For example, the term includes the compounds PBHBV-001 and PBHBV-2-15, and pharmaceutically acceptable salts thereof:



PBHBV-001



PBHBV-2-15

In certain embodiments, the HBV antigen inhibitor is an anti-HBsAg antibody, *e.g.*, mAbs. Certain aspects of the invention are directed to the use of hepatitis B immune globulin (HBIG).

In certain embodiments, the HBV antigen inhibitor is an oligonucleotide. Oligomeric nucleotides can be designed to target one or more genes and/or transcripts of the HBV genome, thereby inhibiting HBV antigen either directly or indirectly. The term "oligonucleotide" or "oligomeric nucleotide" refers to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally- or non-naturally occurring bases, sugars and intersugar (backbone) linkages.

In certain embodiments, the oligonucleotide is an antisense molecule. In certain embodiments, the oligonucleotide is a small interfering RNA (siRNA), Dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA) or microRNA (miRNA).

In certain embodiments, the oligonucleotide is a siRNA molecule. The term "small-interfering RNA" or "siRNA" as used herein refers to double stranded RNA (*i.e.*, duplex RNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.*, by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the siRNA sequence) when the siRNA is in the same cell as the target gene or sequence. The siRNA may have substantial or complete identity to the target gene or sequence, or may comprise a region of mismatch (*i.e.*, a mismatch motif). In certain embodiments, the siRNAs may be about 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length. siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand.

Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see*,

*e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes,* 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.,* 31:981-987 (2003); Knight *et al.*, *Science,* 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.*, 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

In certain embodiments, the siRNA may comprise one or more modified ribonucleotides and/or one or more backbone modifications (e.g., one or more ribonucleotides with a 2'-O-methyl modification, one or more UNA moieties, one or more 2'-Fluoro nucleotides, and/or one or more phosphorothioate linkers).

In certain embodiments, the 5' and/or 3' overhang on one or both strands of the siRNA comprises 1-4 (*e.g.*, 1, 2, 3, or 4) modified and/or unmodified deoxythymidine (t or dT) nucleotides, 1-4 (*e.g.*, 1, 2, 3, or 4) modified (*e.g.*, 2'OMe) and/or unmodified uridine (U) ribonucleotides, and/or 1-4 (*e.g.*, 1, 2, 3, or 4) modified (*e.g.*, 2'OMe) and/or unmodified ribonucleotides or deoxyribonucleotides having complementarity to the target sequence (e.g., 3'overhang in the antisense strand) or the complementary strand thereof (e.g., 3' overhang in the sense strand).

In certain embodiments, the oligonucleotide is an isolated, double stranded, siRNA molecule, that includes a sense strand and an antisense strand that is hybridized to the sense strand. The siRNA target one or more genes and/or transcripts of the HBV genome. In certain embodiments, the HBV inhibitor may be a composition comprising a combination (*e.g.*, a cocktail, pool, or mixture) of siRNAs that target different regions of the HBV genome, and thereby inhibit HBV antigen.

Examples of such siRNA molecules are the siRNA molecules set forth in Tables A and B. Thus, in certain embodiments, the HBV antigen inhibitor is an siRNA molecule selected from the siRNA molecules described in Tables A and B.

These sense and antisense strands, shown in Tables A and B, are useful, for example, for making siRNA molecules that are useful to reduce the expression of one or more HBV genes *in vivo* or *in vitro*. These sense and antisense strands are also useful, for example, as hybridization probes for identifying and measuring the amount of HBV genome in a biological material, such as a tissue or blood sample from a human being infected with HBV or HBV/HDV.

In particular embodiments, an oligonucleotide (such as the sense and antisense RNA strands set forth in Tables A and B) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.,* under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

In certain embodiments, the HBV antigen inhibitor comprises a combination of two or more different siRNA molecules (e.g., a cocktail) selected from the siRNA molecules described in Tables A and B. In certain embodiments, the HBV antigen inhibitor comprises a combination of three or more different siRNA molecules selected from the siRNA molecules described in Tables A and B. Thus, in one aspect, the present invention provides compositions (*e.g.*, pharmaceutical compositions) that include one of the two way or three way combinations of the siRNAs set forth in Tables A and B (*see, e.g.,* Examples 26 and 27). In one aspect, the present invention provides combinations of two or three siRNA molecules, such as the combinations disclosed in the Examples, and compositions comprising such combinations, and uses of such combinations.

In certain embodiments, the HBV antigen inhibitor is a pharmaceutical composition comprising one or more (*e.g.,* a cocktail) of the siRNAs described herein and a pharmaceutically acceptable carrier.

**Table A.**

| siRNA | sense SEQ ID | sense sequence (5'-3') | antisense SEQ ID | antisense sequence (5'-3') |
|---|---|---|---|---|
| 1m | 1 | xArCmCmUrCmUrGrCrCrUrArAmUrCrArUrCrUrCxUrU | 17 | rGrArGrArUrGrArUmUrArGrGmCrArGrArGrGrUxUrU |
| 2m | 2 | xGrCmCmUrCmUrGrCrCrUrArAmUrCrArUrCrUrCxUrU | 18 | rGrArGrArUrGrArUmUrArGrGmCrArGrArGrGrUxUrU |
| 3m | 3 | xArCmCmUrCmUrGrCrCrUrArAmUrCrArUrCrUrGxUrU | 19 | rGrArGrArUrGrArUmUrArGrGmCrArGrArGrGrUxUrU |
| 4m | 4 | xArCmCmUrCmUrGrCrCrUrArAmUrCrArUrCrUrCxUrU | 20 | rCrArGrArUrGrArUmUrArGrGmCrArGrArGrGrUxUrU |
| 5m | 5 | xGrCrGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUxU | 21 | rUrGrArArGrCrGrArArGrUmGrCrArCrArCmGrGxUxU |
| 6m | 6 | xCrCrGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrUxUxU | 22 | rUrGrArArGrCrGrArArGrUmGrCrArCrArCmGrGxUxU |
| 7m | 7 | xCrCrGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUxU | 23 | rArGrArArGrCrGrArArGrUmGrCrArCrArCmGrGxUxU |
| 8m | 8 | xCrCrCmGrAmUrCrCrArUrArCmUmGrCrGmGrArArUxU | 24 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 9m | 9 | xGrCrCmGrAmUrCrCrArUrArCmUmGrCrGmGrArUrUxU | 25 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 10m | 10 | xGrCrCmGrAmUrCrCrArUrArCmUmGrCrGmGrArArUxU | 26 | rArUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 11m | 11 | xGmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 27 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArGrUrU |
| 12m | 12 | xCmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrCrUxU | 28 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArGrUrU |
| 13m | 13 | xCmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 29 | rGrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArGrUrU |
| 14m | 14 | xCmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrCrUxU | 30 | rGrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArGrUrU |
| 15m | 15 | xGmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 31 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArCrUrU |
| 16m | 16 | xGmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrCrUxU | 32 | rGrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArCrUrU |
| 17m | 33 | xArCmCmUrCmUrGmCrCrUrArAmUmCrArUrCmUrCxUrU | 70 | rGrAmGrAmUrGrArUmUrArGrGmCrArGrAmGrGrUxUrU |
| 18m | 34 | xArCmCmUrCmUrGmCrCrUrArAmUmCrArUrCmUrCxUrU | 71 | rGrArGrArUrGmArUmUrArGrGmCrAmGrAmGrGrUxUrU |
| 19m | 35 | xArCmCmUrCmUrGmCrCrUrArAmUmCrArUrCmUrCxUrU | 72 | rGrAmGrAmUrGmArUmUrAmGrGmCrAmGrAmGrGrUxUrU |
| 20m | 36 | xAmCrCmUrCmUrGmCrCmUrAmArUmCrArUrCrUrCxUrU | 73 | rGrAmGrAmUrGrArUmUrArGrGmCrArGrAmGrGrUxUrU |
| 21m | 37 | xAmCrCmUrCmUrGmCrCmUrAmArUmCrArUrCrUrCxUrU | 74 | rGrArGrArUrGmArUmUrArGrGmCrAmGrAmGrGrUxUrU |
| 22m | 38 | xAmCrCmUrCmUrGmCrCmUrAmArUmCrArUrCrUrCxUrU | 75 | rGrAmGrAmUrGmArUmUrAmGrGmCrAmGrAmGrGrUxUrU |
| 23m | 39 | xAmCrCmUrCmUrGmCrCmUrAmArUmCrAmUrCmUrCxUrU | 76 | rGrAmGrAmUrGrArUmUrArGrGmCrArGrAmGrGrUxUrU |
| 24m | 40 | xAmCrCmUrCmUrGmCrCmUrAmArUmCrAmUrCmUrCxUrU | 77 | rGrArGrArUrGmArUmUrArGrGmCrAmGrAmGrGrUxUrU |
| 25m | 41 | xAmCrCmUrCmUrGmCrCmUrAmArUmCrAmUrCmUrCxUrU | 78 | rGrAmGrAmUrGmArUmUrAmGrGmCrAmGrAmGrGrUxUrU |
| 26m | 42 | xCrCmGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUxU | 79 | rUrGrArArGrCmGrArArGrUmGrCrArCrArCmGrGxUxU |
| 27m | 43 | xCrCmGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUxU | 80 | rUrGrArArGrCrGrArArGrUmGrCrAmCrArCmGrGxUxU |
| 28m | 44 | xCrCmGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUxU | 81 | rUrGrArAmGrCmGrArArGmUmGrCrAmCrAmCmGrGxUxU |
| 29m | 45 | xCrCmGrUmGmUrGrCrArCrUmUrCmGrCmUmUrCrAxUxU | 82 | rUrGrArArGrCmGrArArGrUmGrCrArCrArCmGrGxUxU |
| 30m | 46 | xCrCmGrUmGmUrGrCrArCrUmUrCmGrCmUmUrCrAxUxU | 83 | rUrGrArArGrCrGrArArGrUmGrCrAmCrArCmGrGxUxU |
| 31m | 47 | xCrCmGrUmGmUrGrCrArCrUmUrCmGrCmUmUrCrAxUxU | 84 | rUrGrArAmGrCmGrArArGmUmGrCrAmCrAmCmGrGxUxU |
| 32m | 48 | xCrCmGrUmGmUrGmCrArCrUmUrCmGrCmUmUrCrAxUxU | 85 | rUrGrArArGrCmGrArArGrUmGrCrArCrArCmGrGxUxU |
| 33m | 49 | xCrCmGrUmGmUrGmCrArCrUmUrCmGrCmUmUrCrAxUxU | 86 | rUrGrArArGrCrGrArArGrUmGrCrAmCrArCmGrGxUxU |
| 34m | 50 | xCrCmGrUmGmUrGmCrArCrUmUrCmGrCmUmUrCrAxUxU | 87 | rUrGrArAmGrCmGrArArGmUmGrCrAmCrAmCmGrGxUxU |
| 35m | 51 | xCrCrGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUrU | 88 | rUrGrArArGrCrGrArArGrUmGrCrArCrArCmGrGxUrU |
| 36m | 52 | xGrCrCmGrAmUrCmCrArUrArCmUmGrCrGmGrArArUxU | 89 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUmCrGmGrCrUrU |
| 37m | 53 | xGrCrCmGrAmUrCmCrArUrArCmUmGrCrGmGrArArUxU | 90 | rUrUrCrCmGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 38m | 54 | xGrCrCmGrAmUrCmCrArUrArCmUmGrCrGmGrArArUxU | 91 | rUrUrCrCmGrCrAmGrUrAmUrGmGrAmUmCrGmGrCrUrU |
| 39m | 55 | xGrCmCmGrAmUrCmCrArUrArCmUmGrCrGmGrArArUxU | 92 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUmCrGmGrCrUrU |
| 40m | 56 | xGrCmCmGrAmUrCmCrArUrArCmUmGrCrGmGrArArUxU | 93 | rUrUrCrCmGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 41m | 57 | xGrCmCmGrAmUrCmCrArUrArCmUmGrCrGmGrArArUxU | 94 | rUrUrCrCmGrCrAmGrUrAmUrGmGrAmUmCrGmGrCrUrU |
| 42m | 58 | xGrCmCmGrAmUrCmCrAmUrArCmUmGrCrGmGrArArUxU | 95 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUmCrGmGrCrUrU |
| 43m | 59 | xGrCmCmGrAmUrCmCrAmUrArCmUmGrCrGmGrArArUxU | 96 | rUrUrCrCmGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |

| siRNA | sense SEQ ID | sense sequence (5'-3') | antisense SEQ ID | antisense sequence (5'-3') |
|---|---|---|---|---|
| 44m | 60 | xGrCmCmGrAmUrCmCrAmUrArCmUmGrCrGmGrArArUxU | 97 | rUrUrCrCmGrCrAmGrUrAmUrGmGrAmUmCrGmGrCrUrU |
| 45m | 61 | xCmUmGmGrCmUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 98 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCmCrArGrUrU |
| 46m | 62 | xCmUmGmGrCmUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 99 | rCrArCrUrAmGrUrArArArCmUmGrAmGrCrCrArGrUrU |
| 47m | 63 | xCmUmGmGrCmUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 100 | rCrAmCrUrAmGrUrArArArCmUmGrAmGrCmCrArGrUrU |
| 48m | 64 | xCmUmGmGrCmUrCrArGrUrUrUrArCmUrAmGmUrGrUxU | 101 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCmCrArGrUrU |
| 49m | 65 | xCmUmGmGrCmUrCrArGrUrUrUrArCmUrAmGmUrGrUxU | 102 | rCrArCrUrAmGrUrArArArCmUmGrAmGrCrCrArGrUrU |
| 50m | 66 | xCmUmGmGrCmUrCrArGrUrUrUrArCmUrAmGmUrGrUxU | 103 | rCrAmCrUrAmGrUrArArArCmUmGrAmGrCmCrArGrUrU |
| 51m | 67 | xCmUmGmGrCmUrCrArGrUrUmUrAmCmUrAmGmUrGrUxU | 104 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCmCrArGrUrU |
| 52m | 68 | xCmUmGmGrCmUrCrArGrUrUmUrAmCmUrAmGmUrGrUxU | 105 | rCrArCrUrAmGrUrArArArCmUmGrAmGrCrCrArGrUrU |
| 53m | 69 | xCmUmGmGrCmUrCrArGrUrUmUrAmCmUrAmGmUrGrUxU | 106 | rCrAmCrUrAmGrUrArArArCmUmGrAmGrCmCrArGrUrU |
| 54m | 107 | xArCmCmUrCmUrGrCrCrUrArAmUrCrArUrCrUrCxUT | 120 | rGrArGrArUrGrArUmUrArGrGmCrArGrArGrGrUxUT |
| 55m | 108 | xAmCrCmUrCmUrGmCrCmUrAmArUmCrArUrCrUrCxUT | 121 | rGrArGrArUrGmArUmUrArGrGmCrAmGrAmGrGrUxUT |
| 56m | 109 | xGmCrCmUrCmUrGmCrCmUrAmArUmCrArUrCrUrCxUrU | 74 | rGrArGrArUrGmArUmUrArGrGmCrAmGrAmGrGrUxUrU |
| 57m | 110 | xGrCrCmGrAmUrCrCrArUrArCmUmGrCrGmGrArATxU | 122 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCTT |
| 58m | 111 | xCmUmGmGrCmUrCrAmGmUrUmUrAmCmUrAmGmUrGrUxU | 123 | rCrArCrUrAmGmUrArArAmCrUmGrAmGrCmCrArGrUrU |
| 59m | 112 | xCmUmGmGrCmUrCrAmGmUrUmUrAmCmUrAmGmUrGrUxU | 124 | rCrArCmUrAmGrUrArArArCmUmGrAmGrCmCrArGrUrU |
| 60m | 113 | xCmUmGmGrCmUrCrAmGmUrUmUrAmCmUrAmGmUrGrUxU | 125 | rCrArCmUrAmGmUrArArAmCmUmGrAmGrCmCrArGrUrU |
| 61m | 114 | xCmUmGmGrCmUrCrArGmUrUmUrAmCmUrAmGmUmGrUxU | 126 | rCrArCrUrAmGmUrArArAmCrUmGrAmGrCmCrArGrUrU |
| 62m | 115 | xCmUmGmGrCmUrCrArGmUrUmUrAmCmUrAmGmUmGrUxU | 127 | rCrArCmUrAmGrUrArArArCmUmGrAmGrCmCrArGrUrU |
| 63m | 116 | xCmUmGmGrCmUrCrArGmUrUmUrAmCmUrAmGmUmGrUxU | 128 | rCrArCmUrAmGmUrArArAmCmUmGrAmGrCmCrArGrUrU |
| 64m | 117 | xCmUmGmGrCmUrCrAmGmUrUmUrAmCmUrAmGmUmGrUxU | 129 | rCrArCrUrAmGmUrArArAmCrUmGrAmGrCmCrArGrUrU |
| 65m | 118 | xCmUmGmGrCmUrCrAmGmUrUmUrAmCmUrAmGmUmGrUxU | 130 | rCrArCmUrAmGrUrArArArCmUmGrAmGrCmCrArGrUrU |
| 66m | 119 | xCmUmGmGrCmUrCrAmGmUrUmUrAmCmUrAmGmUmGrUxU | 131 | rCrArCmUrAmGmUrArArAmCmUmGrAmGrCmCrArGrUrU |
| 67m | 142 | xCrCrGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUxU | 143 | rUrGrArArGrCrGrArArGrUmGrCrArCrArCmGrGxUxU |
| 68m | 132 | rCrCmGrUmGmUrGrCrArCrUmUrCmGrCmUmUrCrArUrU | 133 | rUrGrArAmGrCmGrArArGmUmGrCrAmCrAmCmGrGrUrU |
| 69m | 134 | rGrCmCmGrAmUrCrCrAmUrArCmUmGrCrGmGrArArUrU | 135 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 70m | 136 | xGrCmCmGrAmUrCrCrAmUrArCmUmGrCrGmGrArArUxU | 135 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 71m | 144 | xCmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 145 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArGrUrU |
| 72m | 138 | rCmUmGmGrCmUrCrArGmUrUmUrAmCmUrAmGmUmGrUrU | 126 | rCrArCrUrAmGmUrArArAmCrUmGrAmGrCmCrArGrUrU |
| 73m | 140 | rAmCrCmUrCmUrGmCrCmUrAmArUmCrArUrCrUrCrUrU | 141 | rGrArGrArUrGmArUmUrArGrGmCrAmGrAmGrGrUrUrU |
| 74m | 10 | xGrCrCmGrAmUrCrCrArUrArCmUmGrCrGmGrArArUxU | 24 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU |
| 75m | 146 | xCrArArGrGmUrArUrGrUrUrGmCrCmCrGmUrUrUxUxU | 152 | rArArArCrGrGrGmCrArAmCrAmUrArCrCrUrUrGxUxU |
| 76m | 147 | xGrCmUrCrAmGrUrUrUrArCmUrAmGrUrGrCrCrArUxU | 153 | rUrGrGrCrArCrUrArGrUrArArArCmUrGrArGrCrUrU |
| 77m | 148 | rCmUrGmUrArGmGrCrArUrArArArUmUrGrGrUrCrUrU | 154 | rGrArCrCrArArUrUmUrArUrGrCrCmUrAmCrArGrUrU |
| 78m | 149 | xUrCrUmGrCmGrGrCrGrUrUmUrUrArUmCrAmUrAxUrU | 155 | rUrAmUrGrArUrArArArArCrGmCrCmGrCrArGrAxUrU |
| 79m | 150 | xUmUrUrArCmUrAmGrUrGrCrCrAmUrUrUrGmUrUxUrU | 17 | rGrArGrArUrGrArUmUrArGrGmCrArGrArGrGrUxUrU |
| 80m | 151 | rArCmCmUrCmUrGrCrCrUrArAmUrCrArUrCrUrCrUrU | 156 | rGrArGrArUrGrArUmUrArGrGmCrArGrArGrGrUrUrU |
| rN = RNA of base N | | | | |
| mN = 2'O-methyl modification of base N | | | | |
| xN = unlocked nucleoside analog moiety of base N | | | | |
| T = deoxythymidine | | | | |

**Table B.**

| siRNA Number | Sense strand SEQ ID NO | Sense strand 5' – 3' | Antisense strand SEQ ID NO | Antisense strand 5'-3' |
|---|---|---|---|---|
| 101 | SEQ ID NO:157 | csgsugugCaCUUcgcuucaccu | SEQ ID NO:158 | asGsgugAaGCgaagUgCacacgsgsuUU |
| 102 | SEQ ID NO:159 | usgsCaCUUcgcuucaccu | SEQ ID NO:160 | asGsgugAaGCgaagUgCacascsgU |
| 103 | SEQ ID NO:161 | usgscaCUUcgcuucaccu | SEQ ID NO:162 | asGsgugaagcgaagUgCacascsgU |
| 104 | SEQ ID NO:163 | usgscaCUUCgcuucaccu | SEQ ID NO:164 | asGsgugAagcgaagUgCacascsgU |
| 105 | SEQ ID NO:165 | CscsGuGuGcACUucGcuuCacc | SEQ ID NO:166 | gsGsUgAaGcgAaguGcAcAcGgsusc |
| 106 | SEQ ID NO:167 | cscsguguGcACUucgcuucacc | SEQ ID NO:168 | gsGsugaAgCGaaguGcAcacggsusc |
| 107 | SEQ ID NO:169 | cscsguGuGcAcUucgcuucacc | SEQ ID NO:170 | gsGsugaAgCGaaguGcAcacggsusc |
| 108 | SEQ ID NO:171 | cscsguguGcACUucgcuuCacc | SEQ ID NO:172 | gsGsugaAgCgaaguGcAcacGgsusc |
| 109 | SEQ ID NO:173 | cscsgugugcACUucgcuucacc | SEQ ID NO:174 | gsGsugaagcgaaguGcAcacggsusc |
| 110 | SEQ ID NO:175 | cscsguguGcacuucgcuucacc | SEQ ID NO:176 | gsgsugaAgCGaagugcacacggsusc |
| 111 | SEQ ID NO:177 | CscsGuGuGcACUucGcuuCacc | SEQ ID NO:178 | gsGsUgAaGcgAaguGcAcAcGgsuscUU |
| 112 | SEQ ID NO:179 | cscsguguGcACUucgcuucacc | SEQ ID NO:180 | gsGsugaAgCGaaguGcAcacggsuscUU |
| 113 | SEQ ID NO:181 | cscsguGuGcAcUucgcuucacc | SEQ ID NO:182 | gsGsugaAgCGaaguGcAcacggsuscUU |
| 114 | SEQ ID NO:183 | cscsguguGcACUucgcuuCacc | SEQ ID NO:184 | gsGsugaAgCgaaguGcAcacGgsuscUU |
| 115 | SEQ ID NO:185 | GsusGcACUucGcuuCacc | SEQ ID NO:186 | gsGsUgAaGcgAaguGcAcAcsGsgU |
| 116 | SEQ ID NO:187 | GsusGcACUucGcuuCacc | SEQ ID NO:188 | gsGsUgAaGcgAaguGcAcAcsGsg |
| 117 | SEQ ID NO:189 | GsusGcACUucGcuuCacc | SEQ ID NO:190 | gsGsUgAaGcgAaguGcAcsAscsGsg |
| 118 | SEQ ID NO:191 | CscsGuGuGcACUucGcuuCaca | SEQ ID NO:192 | usGsUgAaGcgAaguGcAcAcGgsusc |
| 119 | SEQ ID NO:193 | CscsGuGuGcACUucGcuuCaca | SEQ ID NO:194 | usGsUgAaGcgAaguGcAcAcGgsuscUU |
| 120 | SEQ ID NO:195 | cscsguguGcACUucgcuucaca | SEQ ID NO:196 | usGsugaAgCGaaguGcAcacggsuscUU |
| 121 | SEQ ID NO:197 | cscsguGuGcAcUucgcuucaca | SEQ ID NO:198 | usGsugaAgCGaaguGcAcacggsuscUU |
| 122 | SEQ ID NO:199 | cscsguguGcACUucgcuuCaca | SEQ ID NO:200 | usGsugaAgCgaaguGcAcacGgsuscUU |
| 123 | SEQ ID NO:201 | cscsgugugcACUucgcuucaca | SEQ ID NO:202 | usGsugaagcgaaguGcAcacggsuscUU |
| 124 | SEQ ID NO:203 | gsusGcACUucgcuucaca | SEQ ID NO:204 | usGsugaAgCGaaguGcAcacsgsgU |
| 125 | SEQ ID NO:205 | gsusgcACUucgcuucaca | SEQ ID NO:206 | usGsugaagcgaaguGcAcacsgsgU |
| 126 | SEQ ID NO:207 | gsusGcaCUucgcuucaca | SEQ ID NO:208 | uGugaagcgaaguGcAcacsgsgU |
| 127 | SEQ ID NO:209 | GsusGcACUucGcuuCaca | SEQ ID NO:210 | usGsUgAaGcgAaguGcAcAcsGsg |
| 128 | SEQ ID NO:211 | uscsgcuuCaCCUcugcacgucg | SEQ ID NO:212 | csGsacgUgCAgaggUgAagcgasasgUU |
| 129 | SEQ ID NO:213 | uscsgcuuCaCCUcugcacguca | SEQ ID NO:214 | usGsacgUgCAgaggUgAagcgasasgUU |
| 130 | SEQ ID NO:215 | uscsgcUuCaCcUcugcacguca | SEQ ID NO:216 | usGsacgUgCAgaggUgAagcgasasgUU |
| 131 | SEQ ID NO:217 | ususCaCCUcugcacguca | SEQ ID NO:218 | usGsacgUgCAgaggUgAagcsgsaU |
| 132 | SEQ ID NO:219 | ususcaCCUcugcacguca | SEQ ID NO:220 | usGsacgugcagaggUgAagcsgsaU |
| 133 | SEQ ID NO:221 | ususCaCCUcugcacguca | SEQ ID NO:222 | usGsacgUgcagaggUgAagcsgsaU |
| 134 | SEQ ID NO:223 | ususuaCuAgUGCcaUuuguuca | SEQ ID NO:224 | usGsAaCaAauGgcaCuAgUaAascsu |

| siRNA Number | Sense strand SEQ ID NO | Sense strand 5' – 3' | Antisense strand SEQ ID NO | Antisense strand 5'-3' |
|---|---|---|---|---|
| 135 | SEQ ID NO:225 | ususuaCuAgUGCcaUuuguuca | SEQ ID NO:226 | usGsAaCaAauGgcaCuAgUaAascsuUU |
| 136 | SEQ ID NO:227 | ususuacuAgUGCcauuuguuca | SEQ ID NO:228 | usGsaacAaAUggcaCuAguaaascsuUU |
| 137 | SEQ ID NO:229 | ususuaCuAgUgCcauuuguuca | SEQ ID NO:230 | usGsaacAaAUggcaCuAguaaascsuUU |
| 2'-O-Methyl nucleotides = lower case | | | | |
| 2'-Fluoro nucleotides = UPPER CASE | | | | |
| Phosphorothioate linker = s | | | | |
| Unmodified = UPPER CASE | | | | |

In certain embodiments, the oligonucleotide is Arrowhead-ARC-520 (see United States Patent Number 8,809,293; and Wooddell CI, et al., *Molecular Therapy,* **2013**, *21,* 5, 973–985).

In another aspect, term oligonucleotide includes siRNA molecules that target GalNAc and REP 2139, REP-2165 (see, e.g., WO 2016/077321, Al-Mathtab et al., PLoS ONE 11(6):e0156667. doi:10.1371/journal.pone.0156667 and Guillot et al., Poster P0556, EASL, 2015).

In certain embodiments, an HBV antigen inhibitor (e.g., an oligonucleotide, such as an siRNA) is comprised within a lipid nanoparticle formulation.

In certain embodiments, an HBV antigen inhibitor (e.g., an oligonucleotide, such as an siRNA) is conjugated to a targeting moiety. Accordingly, in certain embodiments, an HBV antigen inhibitor (e.g., an oligonucleotide, such as an siRNA) is comprised within a compound of formula (I) or compound of formula (XX).

**Description of Additional Therapeutic Agents**

In certain embodiments, methods of the invention further comprise administering at least one additional therapeutic agent. In certain embodiments, the at least one additional therapeutic agent is selected from the group consisting of:

(A) an agent that controls viral replication;

(B) an agent that reduces viral Ags;

(C) an immune enhancer; and

(D) an immune stimulant.

In certain embodiments, a combination of additional therapeutic agents is administered. In certain embodiments, an additional therapeutic agent may be an HBV antigen inhibitor described herein.

## I. Agents that Control Viral Replication

Category I treatments are directed to the use of agents that control, *e.g.*, inhibit, viral replication.

### *A. Reverse Transcriptase Inhibitors*

In certain embodiments, the reverse transcriptase inhibitor is a nucleoside analog.

In certain embodiments, the reverse transcriptase inhibitor is a nucleoside analog reverse-transcriptase inhibitor (NARTI or NRTI).

In certain embodiments, the reverse transcriptase inhibitor is a nucleotide analog reverse-transcriptase inhibitor (NtARTI or NtRTI).

The term reverse transcriptase inhibitor includes, but is not limited to: entecavir, clevudine, telbivudine, lamivudine, adefovir, and tenofovir, tenofovir disoproxil, tenofovir alafenamide, tenofovir disoproxil fumarate, adefovir dipivoxil, (1R,2R,3R,5R)-3-(6-amino-9H-9-purinyl)-2-fluoro-5-(hydroxymethyl)-4-methylenecyclopentan-1-ol (described in U.S. Patent No. 8,816,074), emtricitabine, abacavir, elvucitabine, ganciclovir, lobucavir, famciclovir, penciclovir, amdoxovir and CMX157 (tenofovir exalidex).

The term reverse transcriptase inhibitor includes, but is not limited to, entecavir, lamivudine, and (1R,2R,3R,5R)-3-(6-amino-9H-9-purinyl)-2-fluoro-5-(hydroxymethyl)-4-methylenecyclopentan-1-ol.

The term reverse transcriptase inhibitor includes, but is not limited to a covalently bound phosphoramidate or phosphonamidate moiety of the above-mentioned reverse transcriptase inhibitors, or as described in, for example, U.S. Patent No. 8,816,074, US 2011/0245484 A1, and US 2008/0286230A1.

The term reverse transcriptase inhibitor includes, but is not limited to, nucleotide analogs that comprise a phosphoramidate moiety, such as, methyl ((((1R,3R,4R,5R)-3-(6-amino-9H-purin-9-yl)-4-fluoro-5-hydroxy-2-methylenecyclopentyl)methoxy)(phenoxy)phosphoryl)-(D or L)-alaninate and methyl ((((1R,2R,3R,4R)-3-fluoro-2-hydroxy-5-methylene-4-(6-oxo-1,6-dihydro-9H-purin-9-yl)cyclopentyl)methoxy)(phenoxy)phosphoryl)-(D or L)-alaninate. Also included are the individual diastereomers thereof, which includes, for example, methyl ((R)-(((1R,3R,4R,5R)-3-(6-amino-9H-purin-9-yl)-4-fluoro-5-hydroxy-2-methylenecyclopentyl)methoxy)(phenoxy)phosphoryl)-(D or L)-alaninate and methyl ((S)-(((1R,3R,4R,5R)-3-(6-amino-9H-purin-9-yl)-4-fluoro-5-hydroxy-2-methylenecyclopentyl)methoxy)(phenoxy)phosphoryl)-(D or L)-alaninate.

The term reverse transcriptase inhibitor includes, but is not limited to a phosphonamidate moiety, such as, tenofovir alafenamide, as well as those described in US 2008/0286230 A1.

Methods for preparing stereoselective phosphoramidate or phosphonamidate containing actives are described in, for example, U.S. Patent No. 8,816,074, as well as US 2011/0245484 A1 and US 2008/0286230 A1.

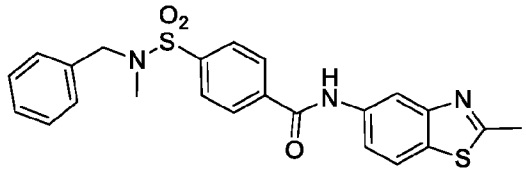
***B. Capsid Inhibitors***

As described herein the term "capsid inhibitor" includes compounds that are capable of inhibiting the expression and/or function of a capsid protein either directly or indirectly. Examples of capsid inhibitors which may be used in methods of the invention are described above.

***C. cccDNA Formation Inhibitors***

Covalently closed circular DNA (cccDNA) is generated in the cell nucleus from viral rcDNA and serves as the transcription template for viral mRNAs. As described herein, the term "cccDNA formation inhibitor" includes compounds that are capable of inhibiting the formation and/or stability of cccDNA either directly or indirectly. For example, a cccDNA formation inhibitor may include, but is not limited to, any compound that inhibits capsid disassembly, rcDNA entry into the nucleus, and/or the conversion of rcDNA into cccDNA. For example, in certain embodiments, the inhibitor detectably inhibits the formation and/or stability of the cccDNA as measured, e.g., using an assay described herein. In certain embodiments, the inhibitor inhibits the formation and/or stability of cccDNA by at least 5%, at least 10%, at least 20%, at least 50%, at least 75%, or at least 90%.

The term cccDNA formation inhibitor includes compounds described in International Patent Application Publication Number WO2013130703, including the following compound:




The term cccDNA formation inhibitor includes, but is not limited to those generally and specifically described in United States Patent Application Publication Number US 2015/0038515 A1. The term cccDNA formation inhibitor includes, but is not limited to, 1-(phenylsulfonyl)-N-(pyridin-4-ylmethyl)-1H-indole-2-carboxamide; 1-Benzenesulfonyl-pyrrolidine-2-carboxylic acid (pyridin-4-ylmethyl)-amide; 2-(2-chloro-N-(2-chloro-5-(trifluoromethyl)phenyl)-4-(trifluoromethyl)phenylsulfonamido)-N-(pyridin-4-ylmethyl)acetamide; 2-(4-chloro-N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-(pyridin-4-ylmethyl)acetamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)-4-

(trifluoromethyl)phenylsulfonamido)-N-(pyridin-4-ylmethyl)acetamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)-4-methoxyphenylsulfonamido)-N-(pyridin-4-ylmethyl)acetamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-((1-methylpiperidin-4-yl)methyl)acetamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-(piperidin-4-ylmethyl)acetamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-(pyridin-4-ylmethyl)propanamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-(pyridin-3-ylmethyl)acetamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-(pyrimidin-5-ylmethyl)acetamide; 2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-(pyrimidin-4-ylmethyl)acetamide; 2-(N-(5-chloro-2-fluorophenyl)phenylsulfonamido)-N-(pyridin-4-ylmethyl)acetamide; 2-[(2-chloro-5-trifluoromethyl-phenyl)-(4-fluoro-benzenesulfonyl)-amino]-N-pyridin-4-ylmethyl-acetamide; 2-[(2-chloro-5-trifluoromethyl-phenyl)-(toluene-4-sulfonyl)-amino]-N-pyridin-4-ylmethyl-acetamide; 2-[benzenesulfonyl-(2-bromo-5-trifluoromethyl-phenyl)-amino]-N-pyridin-4-ylmethyl-acetamide; 2-[benzenesulfonyl-(2-chloro-5-trifluoromethyl-phenyl)-amino]-N-(2-methyl-benzothiazol-5-yl)-acetamide; 2-[benzenesulfonyl-(2-chloro-5-trifluoromethyl-phenyl)-amino]-N-[4-(4-methyl-piperazin-1-yl)-benzyl]-acetamide; 2-[benzenesulfonyl-(2-chloro-5-trifluoromethyl-phenyl)-amino]-N-[3-(4-methyl-piperazin-1-yl)-benzyl]-acetamide; 2-[benzenesulfonyl-(2-chloro-5-trifluoromethyl-phenyl)-amino]-N-benzyl-acetamide; 2-[benzenesulfonyl-(2-chloro-5-trifluoromethyl-phenyl)-amino]-N-pyridin-4-ylmethyl-acetamide; 2-[benzenesulfonyl-(2-chloro-5-trifluoromethyl-phenyl)-amino]-N-pyridin-4-ylmethyl-propionamide; 2-[benzenesulfonyl-(2-fluoro-5-trifluoromethyl-phenyl)-amino]-N-pyridin-4-ylmethyl-acetamide; 4 (N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-N-(pyridin-4-yl- methyl)butanamide; 4-((2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-acetamido)-methyl)-1,1-dimethylpiperidin-1-ium chloride; 4-(benzyl-methyl-sulfamoyl)-N-(2-chloro-5-trifluoromethyl-phenyl)-benzamide; 4-(benzyl-methyl-sulfamoyl)-N-(2-methyl-1H-indol-5-yl)-benzamide; 4-(benzyl-methyl-sulfamoyl)-N-(2-methyl-1H-indol-5-yl)-benzamide; 4-(benzyl-methyl-sulfamoyl)-N-(2-methyl-benzothiazol-5-yl)-benzamide; 4-(benzyl-methyl-sulfamoyl)-N-(2-methyl-benzothiazol-6-yl)-benzamide; 4-(benzyl-methyl-sulfamoyl)-N-(2-methyl-benzothiazol-6-yl)-benzamide; 4-(benzyl-methyl-sulfamoyl)-N-pyridin-4-ylmethyl-benzamide; N-(2-aminoethyl)-2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)-acetamide; N-(2-chloro-5-(trifluoromethyl)phenyl)-N-(2-(3,4-dihydro-2,6-naphthyridin-2(1H)-yl)-2-oxoethyl)benzenesulfonamide; N-benzothiazol-6-yl-4-(benzyl-methyl-sulfamoyl)-benzamide; N-benzothiazol-6-yl-4-(benzyl-methyl-sulfamoyl)-benzamide; tert-butyl (2-(2-(N-(2-chloro-5-

(trifluoromethyl)phenyl)phenylsulfonamido)acetamido)-ethyl)carbamate; and tert-butyl 4-((2-(N-(2-chloro-5-(trifluoromethyl)phenyl)phenylsulfonamido)- acetamido)-methyl)piperidine-1-carboxylate, and optionally, combinations thereof.

***D. Entry Inhibitors***

Certain embodiments of the invention are directed to the use of agents that are HBV entry inhibitors. Entry inhibitors include Myrcludex-B, NTCP inhibitor small molecules, and FXR agonist EYP001 (see, e.g., Gripon, P., Cannie, I. and Urban, S. Efficient Inhibition of Hepatitis B Virus Infection by Acylated Peptides Derived from the Large Viral Surface Protein. Journal of Virology, 79(3): 1613-1622; Volz, T., Allweiss, L., MBarek, M., Warlich, M., Lohse, A., Pollok, J., Alexandrov, A., Urban, S., Petersen, J., Lutgehetmann, M., Dandri, M. The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus. Journal of Hepatology, 58(5): 861-867; Radreau, P., Procherot, M., Vonderscher, J., Lotteau, V., Andre, P. Effect of a novel synthetic FXR agonist EYP001 on hepatitis B virus replication in HepaRG cell line and primary human hepatocytes. AASLD LiverLearning, Abstract 1652, November 16, 2015; WO 2015/036442; WO 00/37077; US2007/0015796). For example, the hepatitis B virus uses its surface lipopeptide pre-S1 for docking to mature liver cells via their sodium/bile acid cotransporter (NTCP) and subsequently entering the cells. Myrcludex B is a synthetic N-acylated pre-S1 that can also dock to NTCP, blocking the virus's entry mechanism.

**II. Agents that Reduce Viral Ags**

Category II treatments are directed to the use of agents that reduce viral antigens.

**A. *Oligomeric Nucleotides***

Examples of oligomeric nucleotides that may be used in methods of the invention are described above (e.g., an HBV antigen inhibitor described herein).

**B. *sAg Secretion Inhibitors***

As described herein, the term "sAg secretion inhibitor" includes compounds that are capable of inhibiting, either directly or indirectly, the secretion of sAg (S, M and/or L surface antigens) bearing subviral particles and/or DNA containing viral particles from HBV-infected cells. Examples of sAg secretion inhibitors that may be used in methods of the invention are described above (e.g., an HBV antigen inhibitor described herein).

***C. Anti-HBsAg agents***

As described herein, the term "anti-HBsAg agents" include anti-HBsAg antibodies, *e.g.*, mAbs. This term also includes hepatitis B immune globulin (HBIG).

**III. Agents that Improve Immune Response**

Category III treatments are directed to the use of agents that improve the immune response against viral infection. In certain embodiments, at least one 'immune enhancer' agent is used in combination with at least one 'immune stimulant agent'. Such a combination can be used in further combination with at least one agent that controls viral replication and/or at least one agent that reduces the viral antigens.

***A. Immune Enhancers***

Certain aspects of the invention are directed to the use of agents that act to improve an immune response by reducing or eliminating immune exhaustion, *e.g.*, by using checkpoint inhibitors, thereby enhancing the immune response.

In certain embodiments, an immune enhancer is a PD-L1 inhibitor. PD-L1 inhibitors are a group of agents that act to inhibit the association of the programmed death-ligand 1 (PD-L1) with its receptor, programmed cell death protein 1 (PD-1).

Immune enhancers include the following:

anti-PD-1 mAbs (*e.g.*, Nivolumab, Pembrolizumab;

anti-PD-L1 mAbs (*e.g.*, Atezolizumab, Avelumab);

anti-PD-L2 mAbs;

anti-CTLA4 mAbs (*e.g.*, Ipilimumab);

anti-VISTA mAbs (*e.g.*, JNJ-61610588);

anti-LAG3 mAbs (*e.g.*, BMS-986016);

anti-TIM3 mAbs (*e.g.*, TSR-022);

peptidomimetics (*e.g.*, AUNP-12); and

small molecule compounds (see, e.g., Zak et al., Oncotarget, 2016, 7(21):30323-35)

***B. Immune Stimulants***

The term "immune stimulant" includes compounds that are capable of modulating an immune response (*e.g.*, stimulating an innate and/or adaptive immune response (*e.g.*, an adjuvant)). The term immune stimulant includes polyinosinic:polycytidylic acid (poly I:C) and interferons.

The term immune stimulant includes agonists of stimulator of IFN genes (STING) and interleukins. The term also includes HBsAg release inhibitors, TLR-7 agonists (GS-9620, RG-7795), T-cell and/or B-cell stimulators (GS-4774, OX-40 agonists (BMS 986178), anti-GITR agonists (BMS-986156)), RIG-1 inhibitors (SB-9200), and SMAC-mimetics (Birinapant).

The term also includes the following:

anti-HBV vaccines (Engerix-B, RECOMBIVAX HB, GS-4744, Heplisav-B);

interferons (Pegylated IFN-α2a, Peglyated IFN-α2b, IFN-α, IFN-λ);

RIG-I agonists (SB-9200);

STING agonists (cGAMP, cGAMP bisphosphorothioate, ADU S100, and other small molecule compounds);

TLR9 agonists (CYT-009, CpG dinucleotides);

TLR7 agonists (GS-9620);

TLR8 agonists (GS-9688);

TLR3 agonists (Ampligen/poly I:C12U);

IL-7 (CYT107); and

IL-2 (aldesleukin).

**Certain Definitions**

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "Hepatitis B virus" (abbreviated as HBV) refers to a virus species of the genus Orthohepadnavirus, which is a part of the Hepadnaviridae family of viruses, and that is capable of causing liver inflammation in humans.

The term "Hepatitis D virus" (abbreviated as HDV) refers to a virus species of the genus Deltaviridae, which is capable of causing liver inflammation in humans. HDV is a small circular enveloped RNA virus that can propagate only in the presence HBV. In particular, HDV requires the HBV surface antigen protein to propagate itself. Infection with both HBV and HDV results in more severe complications compared to infection with HBV alone. These complications include a greater likelihood of experiencing liver failure in acute infections and a rapid progression to liver cirrhosis, with an increased chance of developing liver cancer in chronic infections. In combination with hepatitis B virus, hepatitis D has the highest mortality rate of all the hepatitis infections. The routes of transmission of HDV are similar to those for HBV.

A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product, as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. The term "gene" is used broadly to refer to any segment of nucleic acid associated with a biological function. Genes include coding sequences and/or the regulatory sequences required for their expression. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and

internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions. For example, "gene" refers to a nucleic acid fragment that expresses mRNA, functional RNA, or specific protein, including regulatory sequences. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme RNA, siRNA, or other RNA that may not be translated but yet has an effect on at least one cellular process. "Genes" also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. "Genes" can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters. The term "endogenous gene" refers to a native gene in its natural location in the genome of an organism.

The term "single nucleotide polymorphism (SNP)" refers to a DNA sequence variation that occurs when a single nucleotide in the genome differs between members of a species or paired chromosomes in an individual. As used herein, references to SNPs and SNP genotypes include individual SNPs and/or haplotypes, which are groups of SNPs that are generally inherited together. The disclosure of U.S. Pat. No. 7,820,380, which is incorporated by reference, provides a detailed discussion on a variety of methods that employ SNP genotyping. For example, it is disclosed that various methods for detecting polymorphisms include, but are not limited to, sequencing, methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA duplexes (Myers et al., Science 230: 1242 (1985); Cotton et al., PNAS 85:4397 (1988); and Saleeba et al., Meth. Enzymol. 217:286-295 (1992)), comparison of the electrophoretic mobility of variant and wild type nucleic acid molecules (Orita et al., PNAS 86:2766 (1989); Cotton et al., Mutat. Res. 285: 125-144 (1993); and Hayashi et al., Genet. Anal. Tech. App 9:73-79 (1992)), and assaying the movement of polymorphic or wild type fragments in polyacrylamide gels containing a gradient of denaturant using denaturing gradient gel electrophoresis (DGGE) (Myers et al., Nature 313:495 (1985)). Sequence variations at specific locations can also be assessed by nuclease protection assays such as RNase and 51 protection or chemical cleavage methods. In certain embodiments, sequence variations may be detected using a real-time PCR assay (e.g., employing a fluorescent probe). US. Pat. No. 7,820,380 also discloses that SNP genotyping can include the steps of, for example, collecting a biological sample from a human subject (e.g., sample of tissues, cells, fluids, secretions, etc.), isolating nucleic acids (e.g., genomic DNA, mRNA or both) from the cells of the sample, contacting the nucleic acids with one or more primers which specifically hybridize to a region of the isolated nucleic acid containing a target SNP under conditions such that hybridization and amplification of the target nucleic acid region occurs, and determining the

nucleotide present at the SNP position of interest, or, in some assays, detecting the presence or absence of an amplification product (assays can be designed so that hybridization and/or amplification will only occur if a particular SNP allele is present or absent).

The term "detecting" is used in the broadest sense to include both qualitative and quantitative measurements of a specific molecule, for example, measurements of a specific molecule such as a chromosome, a DNA sequence, individual nucleic acids, etc.

The term "obtaining a biological sample from a hepatitis B patient" is used to refer to obtaining the sample directly from the patient, as well as obtaining the sample indirectly from an intermediary individual (e.g., obtaining the sample from a courier who obtained the sample from a nurse who obtained the sample from the patient).

The term "biological sample" refers to a body sample from any animal, but preferably is from a mammal, more preferably from a human. Such samples include biological fluids such as serum, plasma, vitreous fluid, lymph fluid, synovial fluid, follicular fluid, seminal fluid, amniotic fluid, whole blood, biopsy material, urine, cerebro-spinal fluid, saliva, sputum, tears, perspiration, and mucus. The term also refers to tissue extracts, such as homogenized tissue and cellular extracts, and cellular samples (e.g., oral epithelial cells).

The terms "a higher likelihood of responding to an HBV antigen inhibitor" refers to the likelihood of a patient having a favorable response to the HBV antigen inhibitor. Favorable responses include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In certain embodiments, a favorable response is a reduction in HBV DNA or RNA (e.g., by about 10%, 20%, 30%, 40%, 50%, 60%, 709%, 80%, 90% or more; or e.g., is undetectable after e.g., 1, 2, 3, 4, 5, 6 months, etc. after cessation of HBV therapy). In certain embodiments, a favorable response is sustained virological response (SVR). In certain embodiments, a favorable response is a reduction in HBV core protein (e.g., by about 10%, 20%, 30%, 40%, 50%, 60%, 709%, 80%, 90% or more). In certain embodiments, a favorable response is a reduction in surface antigen (e.g., by about 10%, 20%, 30%, 40%, 50%, 60%, 709%, 80%, 90% or more). In certain embodiments, a favorable response is seroconversion (e.g., HBsAg seroconversion). For example, in certain embodiments, patients that have a C/C genotype at rs12079860 may have a faster rate of surface antigen decline in response to the administration of an HBV antigen inhibitor as compared to a patient having a different genotype. In certain embodiments, patients that have a C/C genotype at rs12079860 may have greater decline in surface antigen in response to the administration of an

HBV antigen inhibitor as compared to a patient having a different genotype. In certain embodiments, patients that have a C/C genotype at rs12079860 may have a higher likelihood of seroconverting in response to an HBV inhibitor as compared to a patient having a different genotype. Methods of measuring a response to an HBV antigen inhibitor are known in the art, for example, an assay described in Example 27 may be used.

As used herein, "treatment" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the typical disease course of the individual being treated. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. In some embodiments, treatments described herein are used to delay development of a disease or to slow the progression of a disease.

The term "patient" as used herein refers to any animal including mammals such as humans, higher non-human primates, rodents domestic and farm animals such as cow, horses, dogs and cats. In one embodiment, the patient is a human patient.

The phrase "effective amount" means an amount of a compound described herein that (i) treats or prevents a hepatitis B virus infection, (ii) attenuates, ameliorates, or eliminates one or more symptoms of a hepatitis B virus infection, or (iii) prevents or delays the onset of one or more symptoms of a hepatitis B virus infection.

The phrase "inhibiting expression of a target gene" refers to the ability of a siRNA described herein to silence, reduce, or inhibit expression of a target gene (*e.g.*, a gene within the HBV genome). To examine the extent of gene silencing, a test sample (*e.g.*, a biological sample from an organism of interest expressing the target gene or a sample of cells in culture expressing the target gene) is contacted with a siRNA that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample is compared to expression of the target gene in a control sample (*e.g.*, a biological sample from an organism of interest expressing the target gene or a sample of cells in culture expressing the target gene) that is not contacted with the siRNA. Control samples (*e.g.*, samples expressing the target gene) may be assigned a value of 100%. In particular embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the value of the test sample relative to the control sample (*e.g.*, buffer only, an siRNA sequence that targets a different gene, a scrambled siRNA sequence, *etc.*) is about 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 70%, 65%, 60%, 55%, 50%,

45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, *e.g.,* dot blots, Northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art. An "effective amount" or "therapeutically effective amount" of a therapeutic nucleic acid such as a siRNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of a siRNA. In particular embodiments, inhibition of expression of a target gene or target sequence is achieved when the value obtained with a siRNA relative to the control (*e.g.,* buffer only, an siRNA sequence that targets a different gene, a scrambled siRNA sequence, *etc.*) is about 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring the expression of a target gene or target sequence include, but are not limited to, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, *e.g.,* dot blots, Northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

The term "nucleic acid" as used herein refers to a polymer containing at least two nucleotides (i.e., deoxyribonucleotides or ribonucleotides) in either single- or double-stranded form and includes DNA and RNA. "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs and/or modified residues include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Additionally, nucleic acids can include one or more UNA moieties.

The term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60 nucleotides generally termed oligonucleotides, and longer fragments termed

polynucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5' and 3' carbons of this sugar to form an alternating, unbranched polymer. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors, expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose. RNA may be in the form, for example, of small interfering RNA (siRNA), Dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Accordingly, in the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.*, 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.*, 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes*, 8:91-98 (1994)).

Described herein are isolated or substantially purified nucleic acid molecules and compositions containing those molecules, which may be used in methods of the invention. In the context of the present invention, an "isolated" or "purified" DNA molecule or RNA molecule is a DNA molecule or RNA molecule that exists apart from its native environment. An isolated DNA molecule or RNA molecule may exist in a purified form or may exist in a non-native environment such as, for example, a transgenic host cell. For example, an "isolated" or "purified" nucleic acid molecule or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In one embodiment, an "isolated" nucleic acid is free of sequences that naturally flank the nucleic acid (*i.e.*, sequences located at the 5′ and 3′ ends of the nucleic acid) in the genomic DNA of the

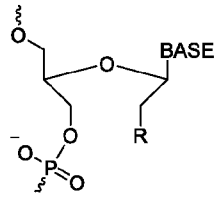
organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived.

The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

The term "unlocked nucleobase analogue" (abbreviated as "UNA") refers to an acyclic nucleobase in which the C2' and C3' atoms of the ribose ring are not covalently linked. The term "unlocked nucleobase analogue" includes nucleobase analogues having the following structure identified as Structure A:

**Structure A**




wherein R is hydroxyl, and Base is any natural or unnatural base such as, for example, adenine (A), cytosine (C), guanine (G) and thymine (T). UNA useful in the practice of the present invention include the molecules identified as acyclic 2'-3'-seco-nucleotide monomers in U.S. patent serial number 8,314,227 which is incorporated by reference herein in its entirety.

The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

The term "lipid particle" includes a lipid formulation that can be used to deliver a therapeutic nucleic acid (*e.g.*, siRNA) to a target site of interest (*e.g.*, cell, tissue, organ, and the like). In preferred embodiments, the lipid particle of the invention is typically formed from a cationic lipid, a non-cationic lipid, and optionally a conjugated lipid that prevents aggregation of the particle. A lipid particle that includes a nucleic acid molecule (*e.g.*, siRNA molecule) is

referred to as a nucleic acid-lipid particle. Typically, the nucleic acid is fully encapsulated within the lipid particle, thereby protecting the nucleic acid from enzymatic degradation.

In certain instances, nucleic acid-lipid particles are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.,* sites physically separated from the administration site), and they can mediate silencing of target gene expression at these distal sites. The nucleic acid may be complexed with a condensing agent and encapsulated within a lipid particle as set forth in PCT Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The lipid particles of the invention typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.,* U.S. Patent Publication Nos. 20040142025 and 20070042031, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

As used herein, "lipid encapsulated" can refer to a lipid particle that provides a therapeutic nucleic acid such as a siRNA, with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid (*e.g.,* siRNA) is fully encapsulated in the lipid particle (*e.g.*, to form a nucleic acid-lipid particle).

The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, PEG-lipid conjugates such as, *e.g.,* PEG coupled to dialkyloxypropyls (*e.g.*, PEG-DAA conjugates), PEG coupled to diacylglycerols (*e.g.*, PEG-DAG conjugates), PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, and PEG conjugated to ceramides (*see, e.g.,* U.S. Patent No. 5,885,613), cationic PEG lipids, polyoxazoline (POZ)-lipid conjugates (*e.g.*, POZ-DAA conjugates), polyamide oligomers (*e.g.,* ATTA-lipid conjugates), and mixtures thereof. Additional examples of POZ-lipid conjugates are described in PCT Publication No. WO 2010/006282. PEG or POZ can be conjugated directly to the lipid or may be linked to the lipid

via a linker moiety. Any linker moiety suitable for coupling the PEG or the POZ to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In certain preferred embodiments, non-ester containing linker moieties, such as amides or carbamates, are used.

The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

The terms "cationic lipid" and "amino lipid" are used interchangeably herein to include those lipids and salts thereof having one, two, three, or more fatty acid or fatty alkyl chains and a pH-titratable amino head group (*e.g.*, an alkylamino or dialkylamino head group). The cationic lipid is typically protonated (*i.e.*, positively charged) at a pH below the $pK_a$ of the cationic lipid and is substantially neutral at a pH above the $pK_a$. The cationic lipids of the invention may also be termed titratable cationic lipids. In some embodiments, the cationic lipids comprise: a protonatable tertiary amine (*e.g.*, pH-titratable) head group; $C_{18}$ alkyl chains, wherein each alkyl chain independently has 0 to 3 (*e.g.*, 0, 1, 2, or 3) double bonds; and ether, ester, or ketal linkages between the head group and alkyl chains. Such cationic lipids include, but are not limited to, DSDMA, DODMA, DLinDMA, DLenDMA, γ-DLenDMA, DLin-K-DMA, DLin-K-C2-DMA (also known as DLin-C2K-DMA, XTC2, and C2K), DLin-K-C3-DMA, DLin-K-C4-DMA, DLen-C2K-DMA, γ-DLen-C2K-DMA, DLin-M-C2-DMA (also known as MC2), and DLin-M-C3-DMA (also known as MC3).

The term "salts" includes any anionic and cationic complex, such as the complex formed between a cationic lipid and one or more anions. Non-limiting examples of anions include inorganic and organic anions, *e.g.*, hydride, fluoride, chloride, bromide, iodide, oxalate (*e.g.*, hemioxalate), phosphate, phosphonate, hydrogen phosphate, dihydrogen phosphate, oxide, carbonate, bicarbonate, nitrate, nitrite, nitride, bisulfite, sulfide, sulfite, bisulfate, sulfate, thiosulfate, hydrogen sulfate, borate, formate, acetate, benzoate, citrate, tartrate, lactate, acrylate, polyacrylate, fumarate, maleate, itaconate, glycolate, gluconate, malate, mandelate, tiglate, ascorbate, salicylate, polymethacrylate, perchlorate, chlorate, chlorite, hypochlorite, bromate, hypobromite, iodate, an alkylsulfonate, an arylsulfonate, arsenate, arsenite, chromate, dichromate, cyanide, cyanate, thiocyanate, hydroxide, peroxide, permanganate, and mixtures thereof. In particular embodiments, the salts of the cationic lipids disclosed herein are crystalline salts.

The term "alkyl" includes a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include, but are not limited to, methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and the like, while saturated branched alkyls include, without limitation, isopropyl, *sec*-butyl,

isobutyl, *tert*-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like, while unsaturated cyclic alkyls include, without limitation, cyclopentenyl, cyclohexenyl, and the like.

The term "alkenyl" includes an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both *cis* and *trans* isomers. Representative straight chain and branched alkenyls include, but are not limited to, ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

The term "alkynyl" includes any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include, without limitation, acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, 3-methyl-1 butynyl, and the like.

The term "acyl" includes any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. The following are non-limiting examples of acyl groups: -C(=O)alkyl, -C(=O)alkenyl, and -C(=O)alkynyl.

The term "heterocycle" includes a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle may be attached via any heteroatom or carbon atom. Heterocycles include, but are not limited to, heteroaryls as defined below, as well as morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

The terms "optionally substituted alkyl", "optionally substituted alkenyl", "optionally substituted alkynyl", "optionally substituted acyl", and "optionally substituted heterocycle" mean that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O), two hydrogen atoms are replaced. In this regard, substituents include, but are not limited to, oxo, halogen, heterocycle, -CN, $-OR^x$, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$, and $-SO_nNR^xR^y$, wherein n is 0, 1, or 2, $R^x$ and $R^y$ are the same or different and are independently hydrogen, alkyl, or heterocycle, and each of the alkyl and heterocycle substituents may be

further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, $-OR^x$, heterocycle, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$, and $-SO_nNR^xR^y$. The term "optionally substituted," when used before a list of substituents, means that each of the substituents in the list may be optionally substituted as described herein.

The term "halogen" includes fluoro, chloro, bromo, and iodo.

The term "fusogenic" refers to the ability of a lipid particle to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

The term "electron dense core", when used to describe a lipid particle described herein, refers to the dark appearance of the interior portion of a lipid particle when visualized using cryo transmission electron microscopy ("cyroTEM"). Some lipid particles of the present invention have an electron dense core and lack a lipid bilayer structure. Some lipid particles of the present invention have an electron dense core, lack a lipid bilayer structure, and have an inverse Hexagonal or Cubic phase structure. While not wishing to be bound by theory, it is thought that the non-bilayer lipid packing provides a 3-dimensional network of lipid cylinders with water and nucleic acid on the inside, i.e., essentially a lipid droplet interpenetrated with aqueous channels containing the nucleic acid.

"Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

"Serum-stable" in relation to nucleic acid-lipid particles means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

"Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent such as a siRNA within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration.

Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

"Local delivery," as used herein, refers to delivery of an active agent such as a siRNA directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site, other target site, or a target organ such as the liver, heart, pancreas, kidney, and the like.

The term "virus particle load", as used herein, refers to a measure of the number of virus particles (e.g., HBV and/or HDV) present in a bodily fluid, such as blood. For example, particle load may be expressed as the number of virus particles per milliliter of, e.g., blood. Particle load testing may be performed using nucleic acid amplification based tests, as well as non-nucleic acid-based tests (*see, e.g.,* Puren et al., The Journal of Infectious Diseases, 201:S27-36 (2010)).

The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

**Generating siRNA Molecules**

siRNA can be provided in several forms including, *e.g.*, as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. In some embodiments, siRNA may be produced enzymatically or by partial/total organic synthesis, and modified ribonucleotides can be introduced by *in vitro* enzymatic or organic synthesis. In certain instances, each strand is prepared chemically. Methods of synthesizing RNA molecules are known in the art, *e.g.,* the chemical synthesis methods as described in Verma and Eckstein (1998) or as described herein.

Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,* Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques known in the art, such as those described in Usman *et al.*, *J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al.*, *Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al.*, *Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μmol scale protocol. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection.

**Carrier Systems Containing Therapeutic Nucleic Acids**

**A. Lipid Particles**

In certain aspects, lipid particles comprising one or more oligonucleotides (*e.g.*, one or more siRNA molecules, such as one or more siRNA molecules described in Tables A and B) and one or more of cationic (amino) lipids or salts thereof, may be administered. In some embodiments, the lipid particles described herein further comprise one or more non-cationic lipids. In other embodiments, the lipid particles further comprise one or more conjugated lipids capable of reducing or inhibiting particle aggregation. The lipid particles of the invention are useful, for example, for delivering a therapeutically effective amount of siRNA into cells (*e.g.*, liver cells) of a human body infected with HBV or HBV/HDV, thereby treating the HBV infection and/or HDV infection and/or ameliorating one or more symptoms of HBV infection and/or HDV infection.

With respect to formulations that include a cocktail of siRNAs encapsulated within lipid particles, the different siRNA molecules may be co-encapsulated in the same lipid particle, or each type of siRNA species present in the cocktail may be encapsulated in its own particle, or some siRNA species may be coencapsulated in the same particle while other siRNA species are encapsulated in different particles within the formulation. Typically, the siRNA molecules of the invention are fully encapsulated in the lipid particle.

The lipid particles described herein preferably comprise one or more siRNA (*e.g.*, siRNA molecules described in Tables A and B), a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the siRNA molecule is fully encapsulated within the lipid portion of the lipid particle such that the siRNA molecule in the lipid particle is resistant in aqueous solution to nuclease degradation. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm. In certain embodiments, the lipid particles have a median diameter of from about 30 nm to about 150 nm. The lipid particles also typically have a lipid:nucleic acid ratio (*e.g.*, a lipid:siRNA ratio) (mass/mass ratio) of from about 1:1 to about 100:1, from about 1:1 to about 50:1, from about 2:1 to about 25:1, from about 3:1 to about 20:1, from about 5:1 to about 15:1, or from about 5:1 to about 10:1. In certain embodiments, the nucleic acid-lipid particle has a lipid:siRNA mass ratio of from about 5:1 to about 15:1.

In preferred embodiments, the lipid particles are serum-stable nucleic acid-lipid particles which comprise one or more siRNA molecules (*e.g.*, a siRNA molecule as described in Tables A and B), a cationic lipid (*e.g.*, one or more cationic lipids of Formula ZI-ZIII or salts thereof as set forth herein), a non-cationic lipid (*e.g.*, mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.*, one or more PEG-lipid conjugates). The lipid particle may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more siRNA molecules (e.g., siRNA molecules described in Tables A and B) that target one or more of the genes described herein. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.*, U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

In the nucleic acid-lipid particles described herein, the one or more siRNA molecules (*e.g.*, an siRNA molecule as described in Tables A and B) may be fully encapsulated within the lipid portion of the particle, thereby protecting the siRNA from nuclease degradation. In certain instances, the siRNA in the nucleic acid-lipid particle is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the siRNA in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In

other embodiments, the siRNA is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the nucleic acid-lipid particle compositions are substantially non-toxic to mammals such as humans.

The term "fully encapsulated" indicates that the siRNA (*e.g.*, a siRNA molecule as described in Tables A and B) in the nucleic acid-lipid particle is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free DNA or RNA. In a fully encapsulated system, preferably less than about 25% of the siRNA in the particle is degraded in a treatment that would normally degrade 100% of free siRNA, more preferably less than about 10%, and most preferably less than about 5% of the siRNA in the particle is degraded. "Fully encapsulated" also indicates that the nucleic acid-lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

In the context of nucleic acids, full encapsulation may be determined by performing a membrane-impermeable fluorescent dye exclusion assay, which uses a dye that has enhanced fluorescence when associated with nucleic acid. Specific dyes such as OliGreen® and RiboGreen® (Invitrogen Corp.; Carlsbad, CA) are available for the quantitative determination of plasmid DNA, single-stranded deoxyribonucleotides, and/or single- or double-stranded ribonucleotides. Encapsulation is determined by adding the dye to a liposomal formulation, measuring the resulting fluorescence, and comparing it to the fluorescence observed upon addition of a small amount of nonionic detergent. Detergent-mediated disruption of the liposomal bilayer releases the encapsulated nucleic acid, allowing it to interact with the membrane-impermeable dye. Nucleic acid encapsulation may be calculated as $E = (I_o - I)/I_o$, where $I$ and $I_o$ refer to the fluorescence intensities before and after the addition of detergent (*see,* Wheeler *et al.*, *Gene Ther.*, 6:271-281 (1999)).

In other embodiments, the present invention provides a nucleic acid-lipid particle composition comprising a plurality of nucleic acid-lipid particles.

In some instances, the nucleic acid-lipid particle composition comprises a siRNA molecule that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%,

from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the particles have the siRNA encapsulated therein.

In other instances, the nucleic acid-lipid particle composition comprises siRNA that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the input siRNA is encapsulated in the particles.

Depending on the intended use of the lipid particles of the invention, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.,* an endosomal release parameter (ERP) assay.

The nucleic acid-lipid particles described herein are useful for the prophylactic or therapeutic delivery, into a human infected with HBV or HBV/HDV, of siRNA molecules that silence the expression of one or more HBV genes, thereby ameliorating at least one symptom of HBV infection and/or HDV infection in the human. In some embodiments, one or more of the siRNA molecules described herein are formulated into nucleic acid-lipid particles, and the particles are administered to a mammal (*e.g.,* a human) requiring such treatment. In certain instances, a therapeutically effective amount of the nucleic acid-lipid particle can be administered to the mammal, (*e.g.,* for treating HBV and/or HDV infection in a human being). The nucleic acid-lipid particles described herein are particularly useful for targeting liver cells in humans which is the site of most HBV gene expression. Administration of the nucleic acid-lipid particle can be by any route known in the art, such as, *e.g.,* oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, or intradermal. In particular embodiments, the nucleic acid-lipid particle is administered systemically, *e.g.,* via enteral or parenteral routes of administration.

In some embodiments, downregulation of HBV gene expression is determined by detecting HBV RNA or protein levels in a biological sample from a mammal after nucleic acid-lipid particle administration. In other embodiments, downregulation of HBV gene expression is determined by detecting HBV mRNA or protein levels in a biological sample from a mammal after nucleic acid-lipid particle administration. In certain embodiments, downregulation of HBV gene expression is detected by monitoring symptoms associated with HBV infection in a mammal after particle administration.

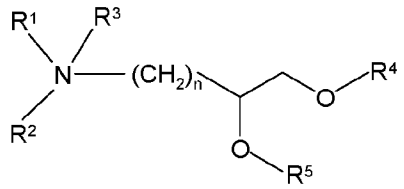
**1. Cationic Lipids**

Any of a variety of cationic lipids or salts thereof may be used in the lipid particles of the present invention either alone or in combination with one or more other cationic lipid species or non-cationic lipid species. The cationic lipids include the (R) and/or (S) enantiomers thereof.

In one aspect of the invention, the cationic lipid is a dialkyl lipid. For example, dialkyl lipids may include lipids that comprise two saturated or unsaturated alkyl chains, wherein each of the alkyl chains may be substituted or unsubstituted. In certain embodiments, each of the two alkyl chains comprise at least, e.g., 8 carbon atoms, 10 carbon atoms, 12 carbon atoms, 14 carbon atoms, 16 carbon atoms, 18 carbon atoms, 20 carbon atoms, 22 carbon atoms or 24 carbon atoms.

In one aspect of the invention, the cationic lipid is a trialkyl lipid. For example, trialkyl lipids may include lipids that comprise three saturated or unsaturated alkyl chains, wherein each of the alkyl chains may be substituted or unsubstituted. In certain embodiments, each of the three alkyl chains comprise at least, e.g., 8 carbon atoms, 10 carbon atoms, 12 carbon atoms, 14 carbon atoms, 16 carbon atoms, 18 carbon atoms, 20 carbon atoms, 22 carbon atoms or 24 carbon atoms.

In one aspect, cationic lipids of Formula ZI having the following structure are useful in the present invention:




(ZI),

or salts thereof, wherein:

$R^1$ and $R^2$ are either the same or different and are independently hydrogen (H) or an optionally substituted $C_1$-$C_6$ alkyl, $C_2$-$C_6$ alkenyl, or $C_2$-$C_6$ alkynyl, or $R^1$ and $R^2$ may join to

form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

$R^3$ is either absent or is hydrogen (H) or a $C_1$-$C_6$ alkyl to provide a quaternary amine;

$R^4$ and $R^5$ are either the same or different and are independently an optionally substituted $C_{10}$-$C_{24}$ alkyl, $C_{10}$-$C_{24}$ alkenyl, $C_{10}$-$C_{24}$ alkynyl, or $C_{10}$-$C_{24}$ acyl, wherein at least one of $R^4$ and $R^5$ comprises at least two sites of unsaturation; and

n is 0, 1, 2, 3, or 4.

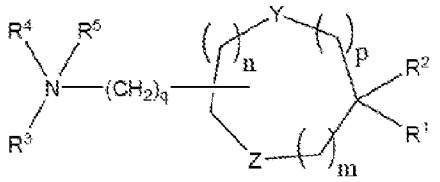
In some embodiments, $R^1$ and $R^2$ are independently an optionally substituted $C_1$-$C_4$ alkyl, $C_2$-$C_4$ alkenyl, or $C_2$-$C_4$ alkynyl. In one preferred embodiment, $R^1$ and $R^2$ are both methyl groups. In other preferred embodiments, n is 1 or 2. In other embodiments, $R^3$ is absent when the pH is above the $pK_a$ of the cationic lipid and $R^3$ is hydrogen when the pH is below the $pK_a$ of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, $R^3$ is an optionally substituted $C_1$-$C_4$ alkyl to provide a quaternary amine. In further embodiments, $R^4$ and $R^5$ are independently an optionally substituted $C_{12}$-$C_{20}$ or $C_{14}$-$C_{22}$ alkyl, $C_{12}$-$C_{20}$ or $C_{14}$-$C_{22}$ alkenyl, $C_{12}$-$C_{20}$ or $C_{14}$-$C_{22}$ alkynyl, or $C_{12}$-$C_{20}$ or $C_{14}$-$C_{22}$ acyl, wherein at least one of $R^4$ and $R^5$ comprises at least two sites of unsaturation.

In certain embodiments, $R^4$ and $R^5$ are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, an arachidonyl moiety, and a docosahexaenoyl moiety, as well as acyl derivatives thereof (*e.g.*, linoleoyl, linolenoyl, γ-linolenoyl, *etc.*). In some instances, one of $R^4$ and $R^5$ comprises a branched alkyl group (*e.g.*, a phytanyl moiety) or an acyl derivative thereof (*e.g.*, a phytanoyl moiety). In certain instances, the octadecadienyl moiety is a linoleyl moiety. In certain other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ-linolenyl moiety. In certain embodiments, $R^4$ and $R^5$ are both linoleyl moieties, linolenyl moieties, or γ-linolenyl moieties. In particular embodiments, the cationic lipid of Formula ZI is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-dilinoleyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLinDMA), 1,2-dilinoleoyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLinDAP), or mixtures thereof.

In some embodiments, the cationic lipid of Formula ZI forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula ZI is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

The synthesis of cationic lipids such as DLinDMA and DLenDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060083780, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as C2-DLinDMA and C2-DLinDAP, as well as additional cationic lipids, is described in international patent application number WO2011/000106 the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In another aspect, cationic lipids of Formula ZII having the following structure (or salts thereof) are useful in the present invention:



(ZII),

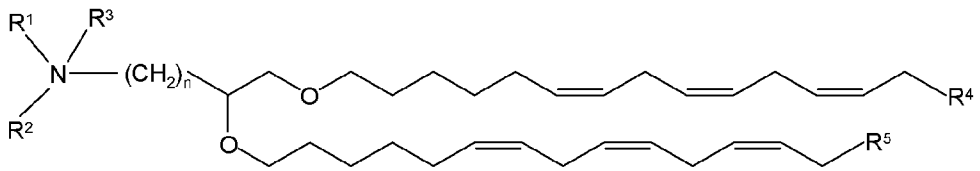
wherein $R^1$ and $R^2$ are either the same or different and are independently an optionally substituted $C_{12}$-$C_{24}$ alkyl, $C_{12}$-$C_{24}$ alkenyl, $C_{12}$-$C_{24}$ alkynyl, or $C_{12}$-$C_{24}$ acyl; $R^3$ and $R^4$ are either the same or different and are independently an optionally substituted $C_1$-$C_6$ alkyl, $C_2$-$C_6$ alkenyl, or $C_2$-$C_6$ alkynyl, or $R^3$ and $R^4$ may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; $R^5$ is either absent or is hydrogen (H) or a $C_1$-$C_6$ alkyl to provide a quaternary amine; m, n, and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and are independently O, S, or NH. In a preferred embodiment, q is 2.

In some embodiments, the cationic lipid of Formula ZII is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2" or "C2K"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA; "C3K"), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA; "C4K"), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-dioleoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DO-K-DMA), 2,2-distearoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DS-K-DMA), 2,2-dilinoleyl-4-N-morpholino-[1,3]-dioxolane (DLin-K-MA), 2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane chloride (DLin-K-TMA.Cl), 2,2-dilinoleyl-4,5-bis(dimethylaminomethyl)-[1,3]-dioxolane (DLin-$K^2$-DMA), 2,2-dilinoleyl-4-methylpiperzine-[1,3]-dioxolane (D-Lin-K-N-methylpiperzine), or mixtures thereof. In preferred embodiments, the cationic lipid of Formula ZII is DLin-K-C2-DMA.

In some embodiments, the cationic lipid of Formula ZII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula ZII is the oxalate (*e.g.,* hemioxalate) salt thereof, which is preferably a crystalline salt.

The synthesis of cationic lipids such as DLin-K-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DLin-K-C2-DMA, DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, DLin-K-MPZ, DO-K-DMA, DS-K-DMA, DLin-K-MA, DLin-K-TMA.Cl, DLin-K$^2$-DMA, and D-Lin-K-N-methylpiperzine, as well as additional cationic lipids, is described in PCT Application No. PCT/US2009/060251, entitled "Improved Amino Lipids and Methods for the Delivery of Nucleic Acids," filed October 9, 2009, the disclosure of which is incorporated herein by reference in its entirety for all purposes.

In a further aspect, cationic lipids of Formula ZIII having the following structure are useful in the present invention:




(ZIII)

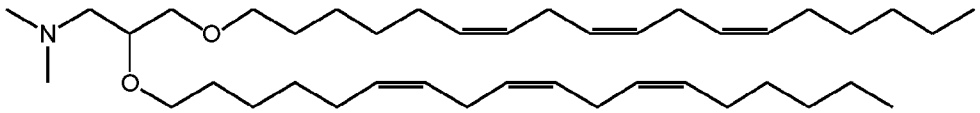
or salts thereof, wherein: $R^1$ and $R^2$ are either the same or different and are independently an optionally substituted $C_1$-$C_6$ alkyl, $C_2$-$C_6$ alkenyl, or $C_2$-$C_6$ alkynyl, or $R^1$ and $R^2$ may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; $R^3$ is either absent or is hydrogen (H) or a $C_1$-$C_6$ alkyl to provide a quaternary amine; $R^4$ and $R^5$ are either absent or present and when present are either the same or different and are independently an optionally substituted $C_1$-$C_{10}$ alkyl or $C_2$-$C_{10}$ alkenyl; and n is 0, 1, 2, 3, or 4.

In some embodiments, $R^1$ and $R^2$ are independently an optionally substituted $C_1$-$C_4$ alkyl, $C_2$-$C_4$ alkenyl, or $C_2$-$C_4$ alkynyl. In a preferred embodiment, $R^1$ and $R^2$ are both methyl groups. In another preferred embodiment, $R^4$ and $R^5$ are both butyl groups. In yet another preferred embodiment, n is 1. In other embodiments, $R^3$ is absent when the pH is above the $pK_a$ of the cationic lipid and $R^3$ is hydrogen when the pH is below the $pK_a$ of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, $R^3$ is an optionally substituted $C_1$-$C_4$ alkyl to provide a quaternary amine. In further embodiments, $R^4$ and $R^5$ are independently an optionally substituted $C_2$-$C_6$ or $C_2$-$C_4$ alkyl or $C_2$-$C_6$ or $C_2$-$C_4$ alkenyl.

In an alternative embodiment, the cationic lipid of Formula ZIII comprises ester linkages between the amino head group and one or both of the alkyl chains. In some embodiments, the cationic lipid of Formula ZIII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula ZIII is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

Although each of the alkyl chains in Formula ZIII contains *cis* double bonds at positions 6, 9, and 12 (*i.e.*, *cis,cis,cis*-$\Delta^6,\Delta^9,\Delta^{12}$), in an alternative embodiment, one, two, or three of these double bonds in one or both alkyl chains may be in the *trans* configuration.

In a particularly preferred embodiment, the cationic lipid of Formula ZIII has the structure:



γ-DLenDMA (**515**)

The synthesis of cationic lipids such as γ-DLenDMA (**515**), as well as additional cationic lipids, is described in U.S. Provisional Application No. 61/222,462, entitled "Improved Cationic Lipids and Methods for the Delivery of Nucleic Acids," filed July 1, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The synthesis of cationic lipids such as DLin-M-C3-DMA ("MC3"), as well as additional cationic lipids (*e.g.*, certain analogs of MC3), is described in U.S. Provisional Application No. 61/185,800, entitled "Novel Lipids and Compositions for the Delivery of Therapeutics," filed June 10, 2009, and U.S. Provisional Application No. 61/287,995, entitled "Methods and Compositions for Delivery of Nucleic Acids," filed December 18, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Examples of other cationic lipids or salts thereof which may be included in the lipid particles of the present invention include, but are not limited to, cationic lipids such as those described in WO2011/000106, the disclosure of which is herein incorporated by reference in its entirety for all purposes, as well as cationic lipids such as N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-

carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-dioeylcarbamoyloxy-3-dimethylaminopropane (DO-C-DAP), 1,2-dimyristoleoyl-3-dimethylaminopropane (DMDAP), 1,2-dioleoyl-3-trimethylaminopropane chloride (DOTAP.Cl), dilinoleylmethyl-3-dimethylaminopropionate (DLin-M-C2-DMA; also known as DLin-M-K-DMA or DLin-M-DMA), and mixtures thereof. Additional cationic lipids or salts thereof which may be included in the lipid particles of the present invention are described in U.S. Patent Publication No. 20090023673, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DLin-C-DAP, DLinDAC, DLinMA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLinTMA.Cl, DLinTAP.Cl, DLinMPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DO-C-DAP, DMDAP, DOTAP.Cl, DLin-M-C2-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US2009/060251, entitled "Improved Amino Lipids and Methods for the Delivery of Nucleic Acids," filed October 9, 2009, the disclosure of which is incorporated herein by reference in its entirety for all purposes. The synthesis of a number of other cationic lipids and related analogs has been described in U.S. Patent Nos. 5,208,036;

5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are each herein incorporated by reference in their entirety for all purposes. Additionally, a number of commercial preparations of cationic lipids can be used, such as, *e.g.*, LIPOFECTIN® (including DOTMA and DOPE, available from Invitrogen); LIPOFECTAMINE® (including DOSPA and DOPE, available from Invitrogen); and TRANSFECTAM® (including DOGS, available from Promega Corp.).

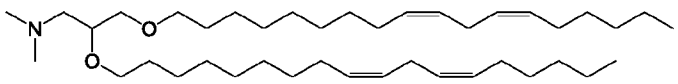
In some embodiments, the cationic lipid comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, from about 50 mol % to about 60 mol %, from about 55 mol % to about 65 mol %, or from about 55 mol % to about 70 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In particular embodiments, the cationic lipid comprises about 50 mol %, 51 mol %, 52 mol %, 53 mol %, 54 mol %, 55 mol %, 56 mol %, 57 mol %, 58 mol %, 59 mol %, 60 mol %, 61 mol %, 62 mol %, 63 mol %, 64 mol %, or 65 mol % (or any fraction thereof) of the total lipid present in the particle.

In other embodiments, the cationic lipid comprises from about 2 mol % to about 60 mol %, from about 5 mol % to about 50 mol %, from about 10 mol % to about 50 mol %, from about 20 mol % to about 50 mol %, from about 20 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, or about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

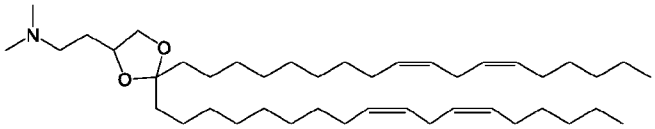
Additional percentages and ranges of cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Published Application No. US 2011/0071208, PCT Publication No. WO2011/000106, and U.S. Published Application No. US 2011/0076335, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

It should be understood that the percentage of cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of cationic lipid present in the formulation may vary, for example, by ± 5 mol %. For example, in one exemplary lipid particle formulation, the target amount of cationic lipid is 57.1 mol %, but the actual amount of cationic lipid may be ± 5 mol %, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle; however, one skilled in the art will understand that the total mol % may deviate slightly from 100% due to rounding, for example, 99.9 mol % or 100.1 mol %.).

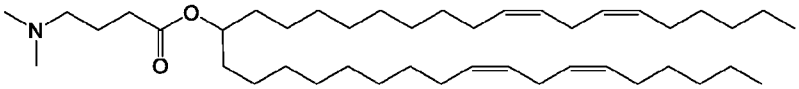
Further examples of cationic lipids useful for inclusion in lipid particles used in the present invention are shown below:



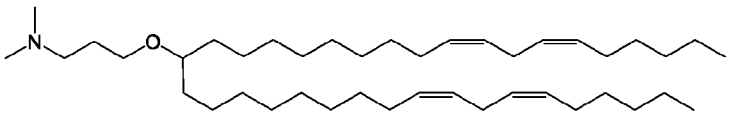
N,N-dimethyl-2,3-bis((9Z,12Z)-octadeca-9,12-dienyloxy)propan-1-amine **(505)**



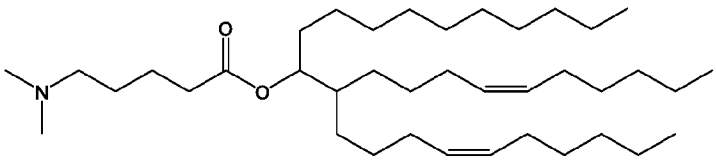
2-(2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-1,3-dioxolan-4-yl)-N,N-dimethylethanamine **(506)**



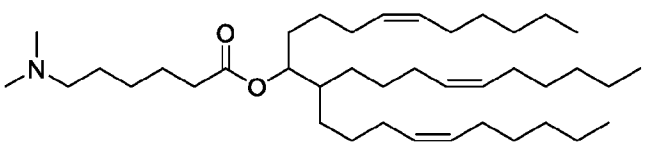
(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate **(507)**



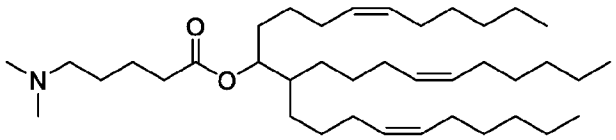
3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine **(508)**



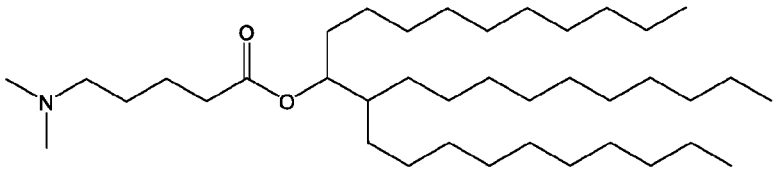
(Z)-12-((Z)-dec-4-enyl)docos-16-en-11-yl 5-(dimethylamino)pentanoate **(553)**



(6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 6-(dimethylamino)hexanoate **(511)**



(6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (**513**)



12-decyldocosan-11-yl 5-(dimethylamino)pentanoate (**514**).

**2. Non-cationic Lipids**

The non-cationic lipids used in the lipid particles of the invention can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used.

The acyl groups in these lipids are preferably acyl groups derived from fatty acids having $C_{10}$-$C_{24}$ carbon chains, *e.g.*, lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof. Non-limiting examples of cholesterol derivatives include polar analogues such as 5α-cholestanol, 5β-coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as 5α-cholestane, cholestenone, 5α-cholestanone, 5β-cholestanone, and cholesteryl decanoate; and mixtures thereof. In preferred embodiments, the cholesterol derivative is a polar analogue such as cholesteryl-(4'-hydroxy)-butyl ether. The synthesis of cholesteryl-(2'-hydroxy)-ethyl ether is described in PCT Publication No. WO 09/127060, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In some embodiments, the non-cationic lipid present in the lipid particles comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof. In other embodiments, the non-cationic lipid present in the lipid particles comprises or consists of one or more phospholipids, *e.g.*, a cholesterol-free lipid particle formulation. In yet other embodiments, the non-cationic lipid present in the lipid particles comprises or consists of cholesterol or a derivative thereof, *e.g.*, a phospholipid-free lipid particle formulation.

Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

In some embodiments, the non-cationic lipid comprises from about 10 mol % to about 60 mol %, from about 20 mol % to about 55 mol %, from about 20 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 50 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 50 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, from about 37 mol % to about 45 mol %, or about 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

In some embodiments, the phospholipid component in the mixture may comprise from about 2 mol % to about 20 mol %, from about 2 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 4 mol % to about 15 mol %, or from about 4 mol % to about 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In an certain embodiments, the phospholipid component in the mixture comprises from about 5 mol % to about 17 mol %, from about 7 mol % to about 17 mol %, from about 7 mol % to about 15 mol %, from about 8 mol % to about 15 mol %, or about 8 mol %, 9 mol %, 10 mol %, 11 mol %, 12 mol %, 13 mol %, 14 mol %, or 15 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof), *e.g.,* in a mixture with cholesterol or a cholesterol derivative at about 34 mol % (or any fraction thereof) of the total lipid present in the particle. As another non-limiting example, a lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof), *e.g.,* in a mixture with cholesterol or a cholesterol derivative at about 32 mol % (or any fraction thereof) of the total lipid present in the particle.

By way of further example, a lipid formulation useful in the practice of the invention has a lipid to drug (*e.g.*, siRNA) ratio of about 10:1 (*e.g.*, a lipid:drug ratio of from 9.5:1 to 11:1, or from 9.9:1 to 11:1, or from 10:1 to 10.9:1). In certain other embodiments, a lipid formulation useful in the practice of the invention has a lipid to drug (*e.g.*, siRNA) ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

In other embodiments, the cholesterol component in the mixture may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 27 mol % to about 37 mol %, from about 25 mol % to about 30 mol %, or from about 35 mol % to about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the cholesterol component in the mixture comprises from about 25 mol % to about 35 mol %, from about 27 mol % to about 35 mol %, from about 29 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 30 mol % to about 34 mol %, from about 31 mol % to about 33 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, or 35 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In embodiments where the lipid particles are phospholipid-free, the cholesterol or derivative thereof may comprise up to about 25 mol %, 30 mol %, 35 mol %, 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

In some embodiments, the cholesterol or derivative thereof in the phospholipid-free lipid particle formulation may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 31 mol % to about 39 mol %, from about 32 mol % to about 38 mol %, from about 33 mol % to about 37 mol %, from about 35 mol % to about 45 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 40 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, or 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a lipid particle formulation may comprise cholesterol at about 37 mol % (or any fraction thereof) of the total lipid present in the particle. As another non-limiting example, a lipid particle formulation may comprise cholesterol at about 35 mol % (or any fraction thereof) of the total lipid present in the particle.

In other embodiments, the non-cationic lipid comprises from about 5 mol % to about 90 mol %, from about 10 mol % to about 85 mol %, from about 20 mol % to about 80 mol %, about 10 mol % (*e.g.*, phospholipid only), or about 60 mol % (*e.g.*, phospholipid and cholesterol or derivative thereof) (or any fraction thereof or range therein) of the total lipid present in the particle.

Additional percentages and ranges of non-cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Published Application No. US 2011/0071208, PCT Publication No. WO2011/000106, and U.S. Published Application No. US 2011/0076335, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

It should be understood that the percentage of non-cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of non-cationic lipid present in the formulation may vary, for example, by ± 5 mol %, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %.

**3. Lipid Conjugates**

In addition to cationic and non-cationic lipids, the lipid particles may further comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, POZ-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures

thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.*, PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.*, U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.*, U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. The disclosures of these patent documents are herein incorporated by reference in their entirety for all purposes.

Additional PEG-lipids suitable for use in the invention include, without limitation, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional suitable PEG-lipid conjugates include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, but are not limited to, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-$NH_2$), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM), as well as such compounds containing a terminal hydroxyl group instead of a terminal methoxy group (*e.g.*, HO-PEG-S, HO-PEG-S-NHS, HO-PEG-$NH_2$, *etc.*). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.*, mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-$CH_2COOH$) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

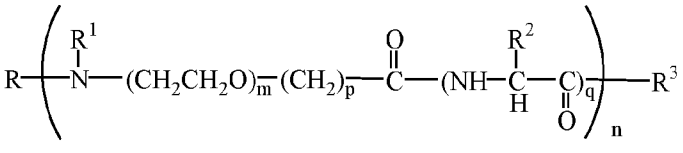
In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-(O)$CCH_2CH_2$C(O)-), succinamidyl (-NHC(O)$CH_2CH_2$C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.*, carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skill in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of $C_{10}$ to $C_{20}$ are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

The term "ATTA" or "polyamide" includes, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated

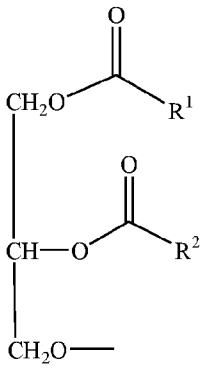
by reference in their entirety for all purposes. These compounds include a compound having the formula:



(ZIV),

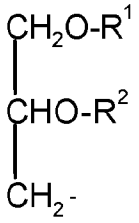
wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; $R^1$ is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and $R^1$ and the nitrogen to which they are bound form an azido moiety; $R^2$ is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; $R^3$ is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and $NR^4R^5$, wherein $R^4$ and $R^5$ are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

The term "diacylglycerol" or "DAG" includes a compound having 2 fatty acyl chains, $R^1$ and $R^2$, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauroyl ($C_{12}$), myristoyl ($C_{14}$), palmitoyl ($C_{16}$), stearoyl ($C_{18}$), and icosoyl ($C_{20}$). In preferred embodiments, $R^1$ and $R^2$ are the same, *i.e.*, $R^1$ and $R^2$ are both myristoyl (*i.e.*, dimyristoyl), $R^1$ and $R^2$ are both stearoyl (*i.e.*, distearoyl), *etc.* Diacylglycerols have the following general formula:



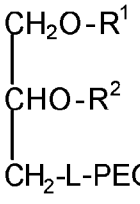
(ZV).

The term "dialkyloxypropyl" or "DAA" includes a compound having 2 alkyl chains, $R^1$ and $R^2$, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(ZVI).

In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



(ZVII),

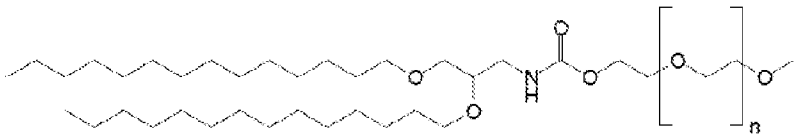
wherein $R^1$ and $R^2$ are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, decyl ($C_{10}$), lauryl ($C_{12}$), myristyl ($C_{14}$), palmityl ($C_{16}$), stearyl ($C_{18}$), and icosyl ($C_{20}$). In preferred embodiments, $R^1$ and $R^2$ are the same, *i.e.*, $R^1$ and $R^2$ are both myristyl (*i.e.*, dimyristyl), $R^1$ and $R^2$ are both stearyl (*i.e.*, distearyl), *etc.*

In Formula ZVII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl groups. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

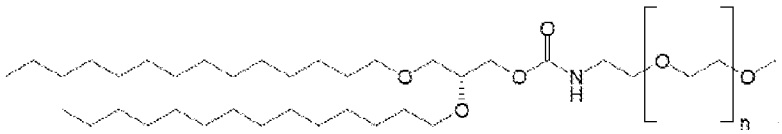
In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-*C*-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-*A*-DAA conjugate). In yet another

preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-*S*-DAA conjugate).

In particular embodiments, the PEG-lipid conjugate is selected from:



(**566**) (PEG-C-DMA); and



(**567**) (PEG-C-DOMG).

The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.*, March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.*, Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

Preferably, the PEG-DAA conjugate is a PEG-didecyloxypropyl ($C_{10}$) conjugate, a PEG-dilauryloxypropyl ($C_{12}$) conjugate, a PEG-dimyristyloxypropyl ($C_{14}$) conjugate, a PEG-dipalmityloxypropyl ($C_{16}$) conjugate, or a PEG-distearyloxypropyl ($C_{18}$) conjugate. In these embodiments, the PEG preferably has an average molecular weight of about 750 or about 2,000 daltons. In one particularly preferred embodiment, the PEG-lipid conjugate comprises PEG2000-C-DMA, wherein the "2000" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In another particularly preferred embodiment, the PEG-lipid conjugate comprises PEG750-C-DMA, wherein the "750" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In particular embodiments, the terminal hydroxyl group of the PEG is substituted with a methyl group. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

In addition to the foregoing components, the lipid particles of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.,* Chen *et al.*, *Bioconj. Chem.*, 11:433-437 (2000); U.S. Patent No. 6,852,334; PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes).

Suitable CPLs include compounds of Formula ZVIII:

A-W-Y (ZVIII),

wherein A, W, and Y are as described below.

With reference to Formula ZVIII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

"W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

"Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between

about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.,* a charge spike. If the charge density is distributed on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.,* U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes), an amide bond will form between the two groups.

In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

In some embodiments, the lipid conjugate (*e.g.,* PEG-lipid) comprises from about 0.1 mol % to about 3 mol %, from about 0.5 mol % to about 3 mol %, or about 0.6 mol %, 0.7 mol %, 0.8 mol %, 0.9 mol %, 1.0 mol %, 1.1 mol %, 1.2 mol %, 1.3 mol %, 1.4 mol %, 1.5 mol %, 1.6 mol %, 1.7 mol %, 1.8 mol %, 1.9 mol %, 2.0 mol %, 2.1 mol%, 2.2 mol%, 2.3 mol %, 2.4

mol %, 2.5 mol %, 2.6 mol %, 2.7 mol %, 2.8 mol %, 2.9 mol % or 3 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In other embodiments, the lipid conjugate (*e.g.*, PEG-lipid) comprises from about 0 mol % to about 20 mol %, from about 0.5 mol % to about 20 mol %, from about 2 mol % to about 20 mol %, from about 1.5 mol % to about 18 mol %, from about 2 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 5 mol % to about 12 mol %, or about 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In further embodiments, the lipid conjugate (*e.g.*, PEG-lipid) comprises from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

It should be understood that the percentage of lipid conjugate present in the lipid particles of the invention is a target amount, and that the actual amount of lipid conjugate present in the formulation may vary, for example, by ± 5 mol %, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %.

Additional percentages and ranges of lipid conjugates suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Published Application No. US 2011/0071208, PCT Publication No. WO2011/000106, and U.S. Published Application No. US 2011/0076335, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the lipid particle is to become fusogenic.

By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the lipid particle and, in turn, the rate at which the lipid particle becomes fusogenic. For instance, when a PEG-DAA conjugate is used as the lipid conjugate, the rate at which the lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the alkyl groups on the PEG-DAA conjugate. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the

lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure. Also, by controlling the composition and concentration of the lipid conjugate, one can control the lipid particle size.

**B. Additional Carrier Systems**

Non-limiting examples of additional lipid-based carrier systems suitable for use in the present invention include lipoplexes (*see, e.g.,* U.S. Patent Publication No. 20030203865; and Zhang *et al.*, *J. Control Release*, 100:165-180 (2004)), pH-sensitive lipoplexes (*see, e.g.,* U.S. Patent Publication No. 20020192275), reversibly masked lipoplexes (*see, e.g.,* U.S. Patent Publication No. 20030180950), cationic lipid-based compositions (*see, e.g.,* U.S. Patent No. 6,756,054; and U.S. Patent Publication No. 20050234232), cationic liposomes (*see, e.g.,* U.S. Patent Publication Nos. 20030229040, 20020160038, and 20020012998; U.S. Patent No. 5,908,635; and PCT Publication No. WO 01/72283), anionic liposomes (*see, e.g.,* U.S. Patent Publication No. 20030026831), pH-sensitive liposomes (*see, e.g.,* U.S. Patent Publication No. 20020192274; and AU 2003210303), antibody-coated liposomes (*see, e.g.,* U.S. Patent Publication No. 20030108597; and PCT Publication No. WO 00/50008), cell-type specific liposomes (*see, e.g.,* U.S. Patent Publication No. 20030198664), liposomes containing nucleic acid and peptides (*see, e.g.,* U.S. Patent No. 6,207,456), liposomes containing lipids derivatized with releasable hydrophilic polymers (*see, e.g.,* U.S. Patent Publication No. 20030031704), lipid-entrapped nucleic acid (*see, e.g.,* PCT Publication Nos. WO 03/057190 and WO 03/059322), lipid-encapsulated nucleic acid (*see, e.g.,* U.S. Patent Publication No. 20030129221; and U.S. Patent No. 5,756,122), other liposomal compositions (*see, e.g.,* U.S. Patent Publication Nos. 20030035829 and 20030072794; and U.S. Patent No. 6,200,599), stabilized mixtures of liposomes and emulsions (*see, e.g.,* EP1304160), emulsion compositions (*see, e.g.,* U.S. Patent No. 6,747,014), and nucleic acid micro-emulsions (*see, e.g.,* U.S. Patent Publication No. 20050037086).

Examples of polymer-based carrier systems suitable for use in the present invention include, but are not limited to, cationic polymer-nucleic acid complexes (*i.e.,* polyplexes). To form a polyplex, a nucleic acid (*e.g.,* a siRNA molecule, such as an siRNA molecule described in Tables A and B) is typically complexed with a cationic polymer having a linear, branched, star, or dendritic polymeric structure that condenses the nucleic acid into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. In some embodiments, the polyplex comprises nucleic acid (*e.g.,* a siRNA molecule, such as an siRNA molecule described in Tables A and B) complexed with a cationic polymer such as polyethylenimine (PEI) (*see, e.g.,* U.S. Patent No. 6,013,240; commercially

available from Qbiogene, Inc. (Carlsbad, CA) as *In vivo* jetPEI™, a linear form of PEI), polypropylenimine (PPI), polyvinylpyrrolidone (PVP), poly-L-lysine (PLL), diethylaminoethyl (DEAE)-dextran, poly(β-amino ester) (PAE) polymers (*see, e.g.,* Lynn *et al.*, *J. Am. Chem. Soc.*, 123:8155-8156 (2001)), chitosan, polyamidoamine (PAMAM) dendrimers (*see, e.g.,* Kukowska-Latallo *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:4897-4902 (1996)), porphyrin (*see, e.g.,* U.S. Patent No. 6,620,805), polyvinylether (*see, e.g.,* U.S. Patent Publication No. 20040156909), polycyclic amidinium (*see, e.g.,* U.S. Patent Publication No. 20030220289), other polymers comprising primary amine, imine, guanidine, and/or imidazole groups (*see, e.g.,* U.S. Patent No. 6,013,240; PCT Publication No. WO/9602655; PCT Publication No. WO95/21931; Zhang *et al.*, *J. Control Release*, 100:165-180 (2004); and Tiera *et al.*, *Curr. Gene Ther.*, 6:59-71 (2006)), and a mixture thereof. In other embodiments, the polyplex comprises cationic polymer-nucleic acid complexes as described in U.S. Patent Publication Nos. 20060211643, 20050222064, 20030125281, and 20030185890, and PCT Publication No. WO 03/066069; biodegradable poly(β-amino ester) polymer-nucleic acid complexes as described in U.S. Patent Publication No. 20040071654; microparticles containing polymeric matrices as described in U.S. Patent Publication No. 20040142475; other microparticle compositions as described in U.S. Patent Publication No. 20030157030; condensed nucleic acid complexes as described in U.S. Patent Publication No. 20050123600; and nanocapsule and microcapsule compositions as described in AU 2002358514 and PCT Publication No. WO 02/096551.

In certain instances, the siRNA may be complexed with cyclodextrin or a polymer thereof. Non-limiting examples of cyclodextrin-based carrier systems include the cyclodextrin-modified polymer-nucleic acid complexes described in U.S. Patent Publication No. 20040087024; the linear cyclodextrin copolymer-nucleic acid complexes described in U.S. Patent Nos. 6,509,323, 6,884,789, and 7,091,192; and the cyclodextrin polymer-complexing agent-nucleic acid complexes described in U.S. Patent No. 7,018,609. In certain other instances, the siRNA may be complexed with a peptide or polypeptide. An example of a protein-based carrier system includes, but is not limited to, the cationic oligopeptide-nucleic acid complex described in PCT Publication No. WO95/21931.

**Preparation of Lipid Particles**

Nucleic acid-lipid particles, in which a nucleic acid (*e.g.,* a siRNA as described in Tables A and B) is entrapped within the lipid portion of the particle and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method, a direct dilution process, and an in-line dilution process.

In particular embodiments, the cationic lipids may comprise lipids of Formula ZI-III or salts thereof, alone or in combination with other cationic lipids. In other embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.*, PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, derivatives thereof, or combinations thereof.

In certain embodiments, the present invention provides nucleic acid-lipid particles produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a siRNA in a first reservoir, providing an organic lipid solution in a second reservoir (wherein the lipids present in the organic lipid solution are solubilized in an organic solvent, *e.g.*, a lower alkanol such as ethanol), and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a lipid vesicle (*e.g.*, liposome) encapsulating the siRNA within the lipid vesicle. This process and the apparatus for carrying out this process are described in detail in U.S. Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a lipid vesicle substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

The nucleic acid-lipid particles formed using the continuous mixing method typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from

about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

In another embodiment, the present invention provides nucleic acid-lipid particles produced via a direct dilution process that includes forming a lipid vesicle (*e.g.*, liposome) solution and immediately and directly introducing the lipid vesicle solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of lipid vesicle solution introduced thereto. As a non-limiting example, a lipid vesicle solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

In yet another embodiment, the present invention provides nucleic acid-lipid particles produced via an in-line dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the lipid vesicle (*e.g.*, liposome) solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the lipid vesicle solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180° (*e.g.*, about 90°). A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of lipid vesicle solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the lipid vesicle solution in the second mixing region, and therefore also the concentration of lipid vesicle solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

These processes and the apparatuses for carrying out these direct dilution and in-line dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The nucleic acid-lipid particles formed using the direct dilution and in-line dilution processes typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

If needed, the lipid particles of the invention can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

In some embodiments, the nucleic acids present in the particles (*e.g.*, the siRNA molecules) are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In other embodiments, the methods may further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brand name POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

In some embodiments, the nucleic acid (*e.g.*, siRNA) to lipid ratios (mass/mass ratios) in a formed nucleic acid-lipid particle will range from about 0.01 to about 0.2, from about 0.05 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials (input) also falls within this range. In other embodiments, the particle preparation uses about 400 µg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 µg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

In other embodiments, the lipid to nucleic acid (*e.g.*, siRNA) ratios (mass/mass ratios) in a formed nucleic acid-lipid particle will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), or about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), 15 (15:1), 16 (16:1), 17 (17:1), 18 (18:1), 19 (19:1), 20 (20:1), 21 (21:1), 22 (22:1), 23 (23:1), 24 (24:1), or 25 (25:1), or any fraction thereof or range therein. The ratio of the starting materials (input) also falls within this range.

As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making lipid particle-CPLs (CPL-containing lipid particles) are discussed herein. Two general techniques include the "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed lipid particle, and the "standard" technique, wherein the

CPL is included in the lipid mixture during, for example, the lipid particle formation steps. The post-insertion technique results in lipid particles having CPLs mainly in the external face of the lipid particle bilayer membrane, whereas standard techniques provide lipid particles having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making lipid particle-CPLs are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

**Description of Certain LNP Embodiments**

As described herein, in certain embodiments, an HBV inhibitor may be an oligonucleotide, such as an siRNA. In certain embodiments, the siRNA may be comprised within a lipid nanoparticle formulation. These nucleic acid-lipid particles may comprise one or more (*e.g.*, a cocktail) of the double-stranded siRNA molecules described herein (*e.g.*, as described in Tables A and B), a cationic lipid, and a non-cationic lipid. In certain instances, the nucleic acid-lipid particles further comprise a conjugated lipid that inhibits aggregation of particles. Preferably, the nucleic acid-lipid particles comprise one or more (*e.g.*, a cocktail) of the double-stranded siRNA molecules described herein, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles.

In certain embodiments, the nucleic acid-lipid particle comprises two different double stranded siRNA molecules.

In certain embodiments, the nucleic acid-lipid particle comprises three different double stranded siRNA molecules.

In some embodiments, the siRNAs are fully encapsulated in the nucleic acid-lipid particle. With respect to formulations comprising an siRNA cocktail, the different types of siRNA species present in the cocktail (*e.g.*, siRNA compounds with different sequences) may be co-encapsulated in the same particle, or each type of siRNA species present in the cocktail may be encapsulated in a separate particle. The siRNA cocktail may be formulated in the particles described herein using a mixture of two, three or more individual siRNAs (each having a unique sequence) at identical, similar, or different concentrations or molar ratios. In one embodiment, a cocktail of siRNAs (corresponding to a plurality of siRNAs with different sequences) is formulated using identical, similar, or different concentrations or molar ratios of each siRNA species, and the different types of siRNAs are co-encapsulated in the same particle. In another embodiment, each type of siRNA species present in the cocktail is encapsulated in different

particles at identical, similar, or different siRNA concentrations or molar ratios, and the particles thus formed (each containing a different siRNA payload) are administered separately (*e.g.*, at different times in accordance with a therapeutic regimen), or are combined and administered together as a single unit dose (*e.g.*, with a pharmaceutically acceptable carrier). The particles described herein are serum-stable, are resistant to nuclease degradation, and are substantially non-toxic to mammals such as humans.

The cationic lipid in the nucleic acid-lipid particles of the invention may comprise, *e.g.*, one or more cationic lipids of Formula ZI-III described herein or any other cationic lipid species. In one embodiment, cationic lipid is a dialkyl lipid. In another embodiment, the cationic lipid is a trialkyl lipid. In one particular embodiment, the cationic lipid is selected from the group consisting of 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), dilinoleylmethyl-3-dimethylaminopropionate (DLin-M-C2-DMA), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-M-C3-DMA; Compound (**507**)), salts thereof, and mixtures thereof.

In another particular embodiment, the cationic lipid is selected from the group consisting of 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)), 3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (DLin-MP-DMA; Compound (**508**)), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) (Compound (**507**)), (6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)), a salt thereof, or a mixture thereof.

In certain embodiments, the cationic lipid comprises from about 48 mol % to about 62 mol % of the total lipid present in the particle.

The non-cationic lipid in the nucleic acid-lipid particles may comprise, *e.g.*, one or more anionic lipids and/or neutral lipids. In some embodiments, the non-cationic lipid comprises one of the following neutral lipid components: (1) a mixture of a phospholipid and cholesterol or a derivative thereof; (2) cholesterol or a derivative thereof; or (3) a phospholipid. In certain preferred embodiments, the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof. In a preferred embodiment, the

non-cationic lipid is a mixture of DPPC and cholesterol. In a preferred embodiment, the non-cationic lipid is a mixture of DSPC and cholesterol.

In certain embodiments, the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from about 7 mol % to about 17 mol % of the total lipid present in the particle and the cholesterol or derivative thereof comprises from about 25 mol % to about 40 mol % of the total lipid present in the particle.

The lipid conjugate in the nucleic acid-lipid particles inhibits aggregation of particles and may comprise, *e.g.,* one or more of the lipid conjugates described herein. In one particular embodiment, the lipid conjugate comprises a PEG-lipid conjugate. Examples of PEG-lipid conjugates include, but are not limited to, PEG-DAG conjugates, PEG-DAA conjugates, and mixtures thereof. In certain embodiments, the PEG-lipid conjugate is selected from the group consisting of a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, a PEG-phospholipid conjugate, a PEG-ceramide (PEG-Cer) conjugate, and a mixture thereof. In certain embodiments, the PEG-lipid conjugate is a PEG-DAA conjugate. In certain embodiments, the PEG-DAA conjugate in the lipid particle may comprise a PEG-didecyloxypropyl ($C_{10}$) conjugate, a PEG-dilauryloxypropyl ($C_{12}$) conjugate, a PEG-dimyristyloxypropyl ($C_{14}$) conjugate, a PEG-dipalmityloxypropyl ($C_{16}$) conjugate, a PEG-distearyloxypropyl ($C_{18}$) conjugate, or mixtures thereof. In certain embodiments, wherein the PEG-DAA conjugate is a PEG-dimyristyloxypropyl ($C_{14}$) conjugate. In another embodiment, the PEG-DAA conjugate is a compound **(566)** (PEG-C-DMA) conjugate. In another embodiment, the lipid conjugate comprises a POZ-lipid conjugate such as a POZ-DAA conjugate.

In certain embodiments, the conjugated lipid that inhibits aggregation of particles comprises from about 0.5 mol % to about 3 mol % of the total lipid present in the particle.

In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1.

In certain embodiments, the nucleic acid-lipid particle has a median diameter of from about 30 nm to about 150 nm.

In certain embodiments, the nucleic acid-lipid particle has an electron dense core.

In some embodiments, the nucleic acid-lipid particles comprising: (a) one or more (*e.g.,* a cocktail) siRNA molecules described herein (*e.g.*, *see*, Tables A and B); (b) one or more cationic lipids or salts thereof comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated

lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, see, Tables A and B); (b) a cationic lipid or a salt thereof comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. In one particular embodiment, the formulation is a four-component system comprising about 1.4 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 57.1 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, about 7.1 mol % DPPC (or DSPC), and about 34.3 mol % cholesterol (or derivative thereof).

In another aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, see, Tables A and B); (b) a cationic lipid or a salt thereof comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. In one particular embodiment, the formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 61.5 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, and about 36.9 mol % cholesterol (or derivative thereof).

Additional formulations are described in PCT Publication No. WO 09/127060 and published US patent application publication number US 2011/0071208 A1, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

In other embodiments, the nucleic acid-lipid particles comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, see, Tables A and B); (b) one or more cationic lipids or salts thereof comprising from about 2 mol % to about 50 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 5 mol % to about 90 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 20 mol % of the total lipid present in the particle.

In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, see, Tables A and B); (b) a

cationic lipid or a salt thereof comprising from about 30 mol % to about 50 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 47 mol % to about 69 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 3 mol % of the total lipid present in the particle. In one particular embodiment, the formulation is a four-component system which comprises about 2 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 40 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, about 10 mol % DPPC (or DSPC), and about 48 mol % cholesterol (or derivative thereof).

In further embodiments, the nucleic acid-lipid particles comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, *see*, Tables A and B); (b) one or more cationic lipids or salts thereof comprising from about 50 mol % to about 65 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 25 mol % to about 45 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle.

In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, *see*, Tables A and B); (b) a cationic lipid or a salt thereof comprising from about 50 mol % to about 60 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 35 mol % to about 45 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle. In certain instances, the non-cationic lipid mixture in the formulation comprises: (i) a phospholipid of from about 5 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 35 mol % of the total lipid present in the particle. In one particular embodiment, the formulation is a four-component system which comprises about 7 mol % PEG-lipid conjugate (*e.g.*, PEG750-C-DMA), about 54 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, about 7 mol % DPPC (or DSPC), and about 32 mol % cholesterol (or derivative thereof).

In another aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, see, Tables A and B); (b) a cationic lipid or a salt thereof comprising from about 55 mol % to about 65 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 30 mol % to about 40 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate

comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle. In one particular embodiment, the formulation is a three-component system which is phospholipid-free and comprises about 7 mol % PEG-lipid conjugate (*e.g.*, PEG750-C-DMA), about 58 mol % cationic lipid (*e.g.*, DLin-K-C2-DMA) or a salt thereof, and about 35 mol % cholesterol (or derivative thereof).

Additional embodiments of useful formulations are described in published US patent application publication number US 2011/0076335 A1, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In certain embodiments of the invention, the nucleic acid-lipid particle comprises: (a) one or more (*e.g.*, a cocktail) siRNA molecules described herein (*e.g.*, see, Tables A and B); (b) a cationic lipid or a salt thereof comprising from about 48 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises about 7 mol % to about 17 mol % of the total lipid present in the particle, and wherein the cholesterol or derivative thereof comprises about 25 mol % to about 40 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 0.5 mol % to about 3.0 mol % of the total lipid present in the particle.

Exemplary lipid formulations A-Z are included below.

Exemplary lipid formulation A includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.2%), cationic lipid (53.2%), phospholipid (9.3%), cholesterol (36.4%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (1.2%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (53.2%), the phospholipid is DPPC (9.3%), and cholesterol is present at 36.4%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation A, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation A may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation A may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments,

the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation B which includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (0.8%), cationic lipid (59.7%), phospholipid (14.2%), cholesterol (25.3%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (0.8%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (59.7%), the phospholipid is DSPC (14.2%), and cholesterol is present at 25.3%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation B, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation B may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation B may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation C includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.9%), cationic lipid (52.5%), phospholipid (14.8%), cholesterol (30.8%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (1.9%), the cationic lipid is 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)) (52.5%), the phospholipid is DSPC (14.8%), and cholesterol is present at 30.8%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation C,

which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation C may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation C may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation D includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (0.7%), cationic lipid (60.3%), phospholipid (8.4%), cholesterol (30.5%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (0.7%), the cationic lipid is 3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (DLin-MP-DMA; Compound (**508**) (60.3%), the phospholipid is DSPC (8.4%), and cholesterol is present at 30.5%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation D, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation D may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation D may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation E includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.8%), cationic lipid (52.1%), phospholipid (7.5%), cholesterol (38.5%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75

mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (1.8%), the cationic lipid is (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) (Compound (**507**)) (52.1%), the phospholipid is DPPC (7.5%), and cholesterol is present at 38.5%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation E, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation E may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation E may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary formulation F includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (0.9%), cationic lipid (57.1%), phospholipid (8.1%), cholesterol (33.8%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (0.9%), the cationic lipid is 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)) (57.1%), the phospholipid is DSPC (8.1%), and cholesterol is present at 33.8%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation F, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation F may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation F may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any

fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation G includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.7%), cationic lipid (61.6%), phospholipid (11.2%), cholesterol (25.5%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (1.7%), the cationic lipid is 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)) (61.6%), the phospholipid is DPPC (11.2%), and cholesterol is present at 25.5%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation G, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation G may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation G may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation H includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.1%), cationic lipid (55.0%), phospholipid (11.0%), cholesterol (33.0%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (1.1%), the cationic lipid is (6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)) (55.0%), the phospholipid is DSPC (11.0%), and cholesterol is present at 33.0%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %,

± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation H, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation H may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation H may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation I includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.6%), cationic lipid (53.1%), phospholipid (9.4%), cholesterol (35.0%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (2.6%), the cationic lipid is (6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)) (53.1%), the phospholipid is DSPC (9.4%), and cholesterol is present at 35.0%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation I, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation I may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation I may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation J includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (0.6%), cationic lipid (59.4%), phospholipid (10.2%), cholesterol (29.8%), wherein the actual amounts of the lipids

present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (0.6%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (59.4%), the phospholipid is DPPC (10.2%), and cholesterol is present at 29.8%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation J, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation J may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation J may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation K includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (0.5%), cationic lipid (56.7%), phospholipid (13.1%), cholesterol (29.7%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (0.5%), the cationic lipid is (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) (Compound (**507**)) (56.7%), the phospholipid is DSPC (13.1%), and cholesterol is present at 29.7%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation K, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation K may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation K may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any

fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation L includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.2%), cationic lipid (52.0%), phospholipid (9.7%), cholesterol (36.2%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (2.2%), the cationic lipid is 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)) (52.0%), the phospholipid is DSPC (9.7%), and cholesterol is present at 36.2%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation L, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation L may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation L may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation M includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.7%), cationic lipid (58.4%), phospholipid (13.1%), cholesterol (25.7%), wherein the actual amounts of the lipids present may vary by by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (2.7%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (58.4%), the phospholipid is DPPC (13.1%), and cholesterol is present at 25.7%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide

a nucleic acid-lipid particle based on formulation M, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation M may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation M may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation N includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (3.0%), cationic lipid (53.3%), phospholipid (12.1%), cholesterol (31.5%), wherein the actual amounts of the lipids present may vary by by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (3.0%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (53.3%), the phospholipid is DPPC (12.1%), and cholesterol is present at 31.5%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation N, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation N may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation N may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation O includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.5%), cationic lipid (56.2%), phospholipid (7.8%), cholesterol (34.7%), wherein the actual amounts of the lipids present may vary by by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ±

0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (1.5%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (56.2%), the phospholipid is DPPC (7.8%), and cholesterol is present at 34.7%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation O, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation O may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation O may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation P includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.1%), cationic lipid (48.6%), phospholipid (15.5%), cholesterol (33.8%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (2.1%), the cationic lipid is 3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (DLin-MP-DMA; Compound (**508**)) (48.6%), the phospholipid is DSPC (15.5%), and cholesterol is present at 33.8%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation P, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation P may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation P may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid

particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation Q includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.5%), cationic lipid (57.9%), phospholipid (9.2%), cholesterol (30.3%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (2.5%), the cationic lipid is (6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)) (57.9%), the phospholipid is DSPC (9.2%), and cholesterol is present at 30.3%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation Q, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation Q may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation Q may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation R includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.6%), cationic lipid (54.6%), phospholipid (10.9%), cholesterol (32.8%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (1.6%), the cationic lipid is 3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (Compound (**508**)) (54.6%), the phospholipid is DSPC (10.9%), and cholesterol is present at 32.8%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based

on formulation R, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation R may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation R may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation S includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.9%), cationic lipid (49.6%), phospholipid (16.3%), cholesterol (31.3%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (2.9%), the cationic lipid is (6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)) (49.6%), the phospholipid is DPPC (16.3%), and cholesterol is present at 31.3%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation S, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation S may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation S may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation T includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (0.7%), cationic lipid (50.5%), phospholipid (8.9%), cholesterol (40.0%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75

mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (0.7%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (50.5%), the phospholipid is DPPC (8.9%), and cholesterol is present at 40.0%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation T, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation T may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation T may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation U includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.0%), cationic lipid (51.4%), phospholipid (15.0%), cholesterol (32.6%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (1.0%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (51.4%), the phospholipid is DSPC (15.0%), and cholesterol is present at 32.6%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation U, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation U may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation U may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug

ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation V includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.3%), cationic lipid (60.0%), phospholipid (7.2%), cholesterol (31.5%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound **(567)**) (1.3%), the cationic lipid is 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA) (60.0%), the phospholipid is DSPC (7.2%), and cholesterol is present at 31.5%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation V, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation V may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation V may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.,* a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation W includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (1.8%), cationic lipid (51.6%), phospholipid (8.4%), cholesterol (38.3%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound **(566)**) (1.8%), the cationic lipid is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) (51.6%), the phospholipid is DSPC (8.4%), and cholesterol is present at 38.3%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation W, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle

based on formulation W may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation W may comprise three different siRNA molecules, In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation X includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.4%), cationic lipid (48.5%), phospholipid (10.0%), cholesterol (39.2%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (2.4%), the cationic lipid is 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)) (48.5%), the phospholipid is DPPC (10.0%), and cholesterol is present at 39.2%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation X, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation X may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation X may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation Y includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.6%), cationic lipid (61.2%), phospholipid (7.1%), cholesterol (29.2%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DMA (compound (**566**)) (2.6%), the cationic lipid is

(6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)) (61.2%), the phospholipid is DSPC (7.1%), and cholesterol is present at 29.2%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation Y, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation Y may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation Y may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from 8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Exemplary lipid formulation Z includes the following components (wherein the percentage values of the components are mole percent): PEG-lipid (2.2%), cationic lipid (49.7%), phospholipid (12.1%), cholesterol (36.0%), wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). For example, in one representative embodiment, the PEG-lipid is PEG-C-DOMG (compound (**567**)) (2.2%), the cationic lipid is (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) (Compound (**507**)) (49.7%), the phospholipid is DPPC (12.1%), and cholesterol is present at 36.0%, wherein the actual amounts of the lipids present may vary by, *e.g.*, ± 5 % (or *e.g.*, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol %). Thus, certain embodiments of the invention provide a nucleic acid-lipid particle based on formulation Z, which comprises one or more siRNA molecules described herein. For example, in certain embodiments, the nucleic acid lipid particle based on formulation Z may comprise two different siRNA molecules. In certain other embodiments, the nucleic acid lipid particle based on formulation Z may comprise three different siRNA molecules. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of from about 5:1 to about 15:1, or about 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, or 15:1, or any fraction thereof or range therein. In certain embodiments, the nucleic acid-lipid particle has a total lipid:siRNA mass ratio of about 9:1 (*e.g.*, a lipid:drug ratio of from 8.5:1 to 10:1, or from

8.9:1 to 10:1, or from 9:1 to 9.9:1, including 9.1:1, 9.2:1, 9.3:1, 9.4:1, 9.5:1, 9.6:1, 9.7:1, and 9.8:1).

Accordingly, described herein are nucleic acid-lipid particles, wherein the lipids are formulated as described in any one of formulations A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y or Z.

Also described herein are pharmaceutical compositions comprising a nucleic acid-lipid particle and a pharmaceutically acceptable carrier.

The nucleic acid-lipid particles described herein are useful, for example, for the therapeutic delivery of siRNAs that silence the expression of one or more HBV genes. In some embodiments, a cocktail of siRNAs that target different regions (*e.g.,* overlapping and/or non-overlapping sequences) of an HBV gene or transcript is formulated into the same or different nucleic acid-lipid particles, and the particles are administered to a mammal (*e.g.,* a human) requiring such treatment. In certain instances, a therapeutically effective amount of the nucleic acid-lipid particles can be administered to the mammal, *e.g.,* for treating HBV and/or HDV infection in a human.

In certain embodiments, one or more siRNA molecules described herein may be introduced into a cell by contacting the cell with a nucleic acid-lipid particle described herein.

In certain embodiments, one or more siRNA molecules that silence expression of a Hepatitis B virus gene may be introduced into a cell by contacting the cell with a nucleic acid-lipid particle described herein under conditions whereby the siRNA enters the cell and silences the expression of the Hepatitis B virus gene within the cell. In certain embodiments, the cell is in a mammal, such as a human. In certain embodiments, the human has been diagnosed with a Hepatitis B virus infection or a Hepatitis B virus/Hepatitis D virus infection. In certain embodiments, silencing of the Hepatitis B virus gene expression reduces Hepatitis B virus and/or Hepatitis D virus particle load in the mammal by at least about 50% (*e.g.,* about 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99 or 100%) relative to Hepatitis B virus and/or Hepatitis D virus particle load in the absence of the nucleic acid-lipid particle.

In certain embodiments, the expression of a Hepatitis B virus gene in a cell may be silenced by contacting a cell comprising an expressed Hepatitis B virus gene with a nucleic acid-lipid particle or a composition (e.g., a pharmaceutical composition) described herein under conditions whereby the siRNA enters the cell and silences the expression of the Hepatitis B virus gene within the cell. In certain embodiments, the cell is in a mammal, such as a human. In certain embodiments, the human has been diagnosed with a Hepatitis B virus infection or a Hepatitis B virus/Hepatitis D virus infection. In certain embodiments, the human has been

diagnosed with liver disease caused by a Hepatitis B virus infection or a Hepatitis B virus/Hepatitis D virus infection. In certain embodiments, silencing of the Hepatitis B virus gene expression reduces Hepatitis B virus and/or Hepatitis D virus particle load in the mammal by at least about 50% (*e.g.*, about 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99 or 100%) relative to Hepatitis B virus and/or Hepatitis D virus particle load in the absence of the nucleic acid-lipid particle.

In some embodiments, the nucleic acid-lipid particles or compositions (e.g., a pharmaceutical composition) described herein are administered by one of the following routes of administration: oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. In particular embodiments, the nucleic acid-lipid particles are administered systemically, *e.g.*, via enteral or parenteral routes of administration.

In certain aspects, HBV gene expression may be silenced in a mammal (*e.g.*, human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle comprising one or more siRNAs described herein (*e.g.*, one or more siRNAs shown in Tables A and B). In some embodiments, administration of nucleic acid-lipid particles comprising one or more siRNAs described herein reduces HBV RNA levels by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% (or any range therein) relative to HBV RNA levels detected in the absence of the siRNA (*e.g.*, buffer control or irrelevant non-HBV targeting siRNA control). In other embodiments, administration of nucleic acid-lipid particles comprising one or more HBV-targeting siRNAs reduces HBV RNA levels for at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 days or more (or any range therein) relative to a negative control such as, *e.g.*, a buffer control or an irrelevant non-HBV targeting siRNA control.

In other aspects, HBV gene expression may be silenced in a mammal (*e.g.*, human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle comprising one or more siRNAs described herein (*e.g.*, siRNAs described in Tables A and B). In some embodiments, administration of nucleic acid-lipid particles comprising one or more HBV siRNAs reduces HBV mRNA levels by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% (or any range therein) relative to HBV mRNA levels detected in the absence of the siRNA

(*e.g.*, buffer control or irrelevant non-HBV targeting siRNA control). In other embodiments, administration of nucleic acid-lipid particles comprising one or more HBV-targeting siRNAs reduces HBV mRNA levels for at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 days or more (or any range therein) relative to a negative control such as, *e.g.*, a buffer control or an irrelevant non-HBV targeting siRNA control.

Also described herein are methods for treating, preventing, reducing the risk or likelihood of developing (*e.g.*, reducing the susceptibility to), delaying the onset of, and/or ameliorating one or more symptoms associated with HBV and/or HDV infection in a mammal (*e.g.*, human) in need thereof. In certain embodiments, such methods comprise administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle comprising one or more siRNA molecules described herein (*e.g.*, as described in Tables A and B) that target HBV gene expression. Examples of symptoms associated with HBV and/or HDV infection in a human include fever, abdominal pain, dark urine, joint pain, loss of appetite, nausea, vomiting, weakness, fatigue and yellowing of the skin (jaundice).

In further aspects, HBV and/or HDV may be inactivated in a mammal (*e.g.*, human) in need thereof (*e.g.*, a human suffering from HBV infection or HBV/HDV infection), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle comprising one or more siRNAs described herein that target HBV gene expression. In some embodiments, administration of nucleic acid-lipid particles comprising one or more HBV-targeting siRNAs lowers, reduces, or decreases HBV protein levels (*e.g.*, HBV surface antigen protein) by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% (or any range therein) relative to the HBV protein levels detected in the absence of the siRNA (*e.g.*, buffer control or irrelevant non-HBV targeting siRNA control).

By way of example, HBV mRNA can be measured using a branched DNA assay (QuantiGene®; Affymetrix). The branched DNA assay is a sandwich nucleic acid hybridization method that uses bDNA molecules to amplify signal from captured target RNA.

In addition to its utility in silencing the expression of any of the HBV genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies

directed at testing whether a specific member of the HBV gene family has the potential to be a therapeutic target.

**Administration of Lipid Particles**

Once formed, the lipid particles of the invention are particularly useful for the introduction of a siRNA molecule (*e.g.*, a siRNA molecule as described in Tables A and B) into cells. Accordingly, the present invention also provides methods for introducing a siRNA molecule into a cell. In particular embodiments, the siRNA molecule is introduced into an infected cell. The methods may be carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of siRNA to the cells to occur.

The lipid particles of the invention (*e.g.*, a nucleic-acid lipid particle) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the siRNA portion of the particle can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

The lipid particles of the invention (*e.g.*, nucleic acid-lipid particles) can be administered either alone or in a mixture with a pharmaceutically acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

The pharmaceutically acceptable carrier is generally added following lipid particle formation. Thus, after the lipid particle is formed, the particle can be diluted into pharmaceutically acceptable carriers such as normal buffered saline.

The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol, and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

In some embodiments, the lipid particles of the invention are particularly useful in methods for the therapeutic delivery of one or more siRNA molecules (*e.g.*, an siRNA molecule as described in Tables A and B). In particular, it is an object of this invention to provide *in vivo* methods for treatment of HBV and/or HDV infection in humans by downregulating or silencing the transcription and/or translation of one or more HBV genes.

**A. *In vivo* Administration**

Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a siRNA molecule described herein, such as an siRNA described in Tables A and B, to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453, the disclosures of which are herein incorporated by reference in their entirety for all purposes. The present invention also provides fully encapsulated lipid particles that protect the siRNA from nuclease degradation in serum, are non-immunogenic, are small in size, and are suitable for repeat dosing. Additionally, the one or more siRNA molecules may be administered

alone in the lipid particles of the invention, or in combination (*e.g.,* co-administered) with lipid particles comprising peptides, polypeptides, or small molecules such as conventional drugs.

For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intranasal or intratracheal), transdermal application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see*, *e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques,* 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see*, *e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)). The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

In embodiments where the lipid particles of the present invention are administered intravenously, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the particles is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles is detectable at least about 1 hour after administration of the particle. In some embodiments, the presence of a siRNA molecule is detectable in cells at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence, such as a viral or host sequence, by a siRNA molecule is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence, such as a viral or host sequence, by a siRNA molecule occurs preferentially in infected cells and/or cells capable of being infected. In further embodiments, the presence or effect of a siRNA molecule in cells at a site proximal or distal to the site of administration is detectable at about 12, 24, 48, 72, or 96

hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles of the invention are administered parenterally or intraperitoneally.

The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am. J. Sci.*, 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.*, in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will

be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc*. These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451, the disclosures of which are herein incorporated by reference in their entirety for all purposes). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged siRNA molecule (*e.g.*, a siRNA molecule described in Tables A and B) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a siRNA molecule, as liquids, solids,

granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a siRNA molecule in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic nucleic acid in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the siRNA molecule, carriers known in the art.

In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

When preparing pharmaceutical preparations of the lipid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as siRNA associated with the external surface.

The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

The amount of particles administered will depend upon the ratio of siRNA molecules to lipid, the particular siRNA used, the strain of HBV being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about $10^8$-$10^{10}$ particles per administration (*e.g.*, injection).

**B. *In vitro* Administration**

For *in vitro* applications, the delivery of siRNA molecules can be to any cell grown in culture. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment

of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about $10^3$ to about $10^5$ cells/ml, more preferably about 2 x $10^4$ cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al.*, Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of a nucleic acid-lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829, the disclosure of which is herein incorporated by reference in its entirety for all purposes. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of the lipid particle based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the lipid particle affects delivery efficiency, thereby optimizing the lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a lipid particle formulation optimized for an expression plasmid will also be appropriate for encapsulating a siRNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of a siRNA. By comparing the ERPs for each of the various lipid particles, one can readily determine the optimized system, *e.g.*, the lipid particle that has the greatest uptake in the cell.

**C. Detection of Lipid Particles**

In some embodiments, the lipid particles of the present invention are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g.*, by direct detection of the particles, detection of

a siRNA sequence, detection of the target sequence of interest (*i.e.*, by detecting expression or reduced expression of the sequence of interest), detection of a compound modulated by an EBOV protein (*e.g.*, interferon), detection of viral load in the subject, or a combination thereof.

**1. Detection of Particles**

Lipid particles of the invention can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc.*, digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as $^{3}H$, $^{125}I$, $^{35}S$, $^{14}C$, $^{32}P$, $^{33}P$, *etc.*; enzymes such as horse radish peroxidase, alkaline phosphatase, *etc.*; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

**2. Detection of Nucleic Acids**

Nucleic acids (*e.g.*, siRNA molecules) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g.*, "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

The sensitivity of the hybridization assays may be enhanced through the use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods,

including the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Qβ-replicase amplification, and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; The *Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation. The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of polynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic polynucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

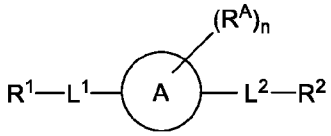
An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or

alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

**Embodiments of Certain Conjugates**

As described herein, an HBV antigen inhibitor may be an oligonucleotide, such as an siRNA molecule. Such molecules (e.g., as described in Tables A and B) may be conjugated to a targeting moiety. For example, in certain embodiments, the oligonucleotide (e.g., siRNA molecule) may be comprised within a compound of formula I or XX.

Accordingly, in one aspect an oligonucleotide is comprised within a compound of formula I:




(I)

wherein:

$R^1$ a is targeting ligand;

$L^1$ is absent or a linking group;

$L^2$ is absent or a linking group;

$R^2$ is an oligonucleotide;

the ring A is absent, a 3-20 membered cycloalkyl, a 5-20 membered aryl, a 5-20 membered heteroaryl, or a 3-20 membered heterocycloalkyl;

each $R^A$ is independently selected from the group consisting of hydrogen, hydroxy, CN, F, Cl, Br, I, -$C_{1-2}$ alkyl-$OR^B$, $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl; wherein the $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl are optionally substituted with one or more groups independently selected from halo, hydroxy, and $C_{1-3}$ alkoxy;

$R^B$ is hydrogen, a protecting group, a covalent bond to a solid support, or a bond to a linking group that is bound to a solid support; and

n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

or a salt thereof.

In one embodiment of the compound of formula I, $R^1$ a is targeting ligand;

$L^1$ is absent or a linking group;

$L^2$ is absent or a linking group;

$R^2$ is an oligonucleotide;

the ring A is absent, a 3-20 membered cycloalkyl, a 5-20 membered aryl, a 5-20 membered heteroaryl, or a 3-20 membered heterocycloalkyl;

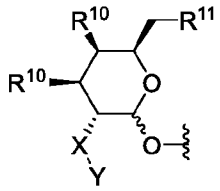
each $R^A$ is independently selected from the group consisting of hydrogen, hydroxy, CN, F, Cl, Br, I, -$C_{1-2}$ alkyl-$OR^B$ and $C_{1-8}$ alkyl that is optionally substituted with one or more groups independently selected from halo, hydroxy, and $C_{1-3}$ alkoxy;

$R^B$ is hydrogen, a protecting group, a covalent bond to a solid support, or a bond to a linking group that is bound to a solid support; and

n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

In one embodiment $R^1$ is $–C(H)_{(3-p)}(L^3\text{-saccharide})_p$, wherein each $L^3$ is independently a linking group; p is 1, 2, or 3; and saccharide is a monosaccharide or disaccharide.

In one embodiment the saccharide is:



wherein:

X is $NR^3$, and Y is selected from -(C=O)$R^4$, -$SO_2R^5$, and -(C=O)$NR^6R^7$; or X is -(C=O)- and Y is $NR^8R^9$;

$R^3$ is hydrogen or ($C_1$-$C_4$)alkyl;

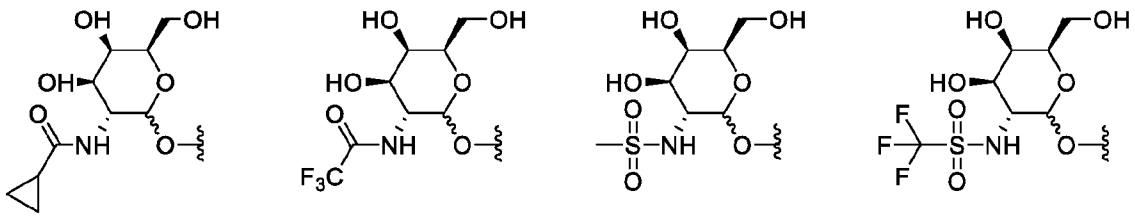
$R^4$, $R^5$, $R^6$, $R^7$ , $R^8$ and $R^9$ are each independently selected from the group consisting of hydrogen, ($C_1$-$C_8$)alkyl, ($C_1$-$C_8$)haloalkyl, ($C_1$-$C_8$)alkoxy and ($C_3$-$C_6$)cycloalkyl that is optionally substituted with one or more groups independently selected from the group consisting of halo, ($C_1$-$C_4$)alkyl, ($C_1$-$C_4$)haloalkyl, ($C_1$-$C_4$)alkoxy and ($C_1$-$C_4$)haloalkoxy;

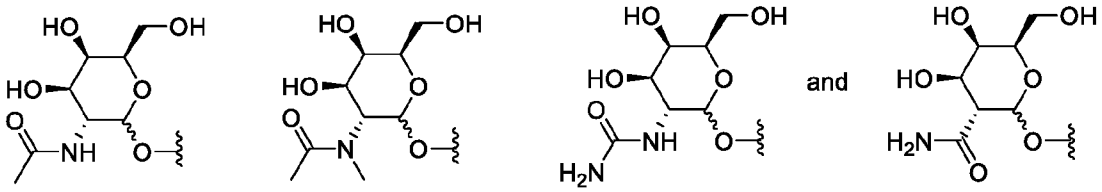
$R^{10}$ is -OH, -$NR^8R^9$ or – F; and

$R^{11}$ is -OH, -$NR^8R^9$, -F or 5 membered heterocycle that is optionally substituted with one or more groups independently selected from the group consisting of halo, hydroxyl, carboxyl, amino, ($C_1$-$C_4$)alkyl, ($C_1$-$C_4$)haloalkyl, ($C_1$-$C_4$)alkoxy and ($C_1$-$C_4$)haloalkoxy;

or a salt thereof.

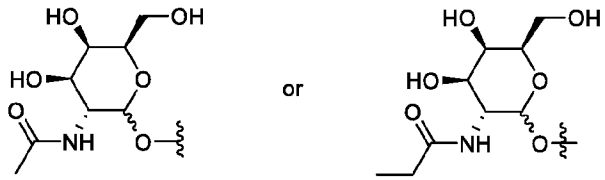
In one embodiment the saccharide is selected from the group consisting of:





and salts thereof.

In one embodiment the saccharide is:

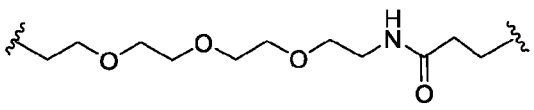


*N*-Acetylgalactosamine (GalNAc) GalPro

In one embodiment each $L^3$ is independently a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 0 to 50 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or ($C_1$-$C_6$)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkanoyl, ($C_1$-$C_6$)alkanoyloxy, ($C_1$-$C_6$)alkoxycarbonyl, ($C_1$-$C_6$)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

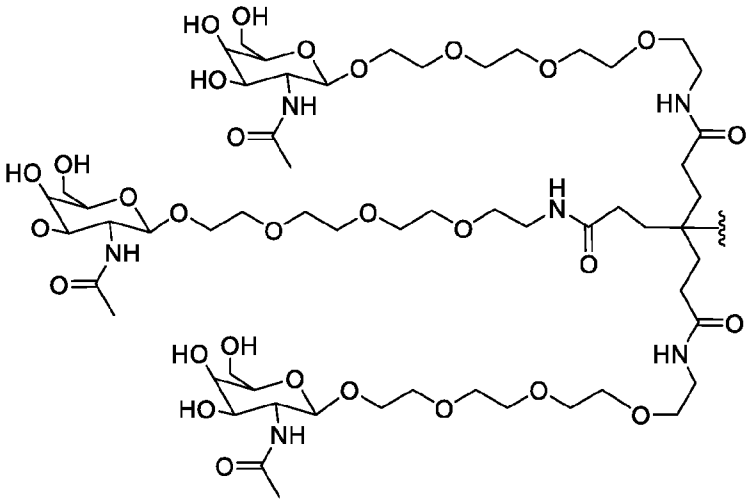
In one embodiment each $L^3$ is independently a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 20 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or ($C_1$-$C_6$)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkanoyl, ($C_1$-$C_6$)alkanoyloxy, ($C_1$-$C_6$)alkoxycarbonyl, ($C_1$-$C_6$)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

In one embodiment $L^3$ is:



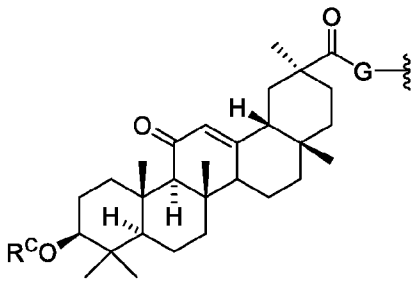
or a salt thereof.

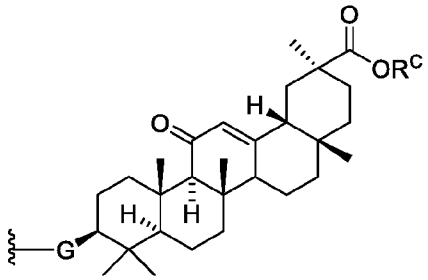
In one embodiment $R^1$ is:



or a salt thereof.

In one embodiment $R^1$ is:



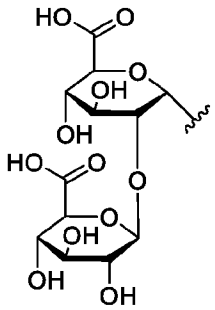


wherein G is –NH- or –O-;

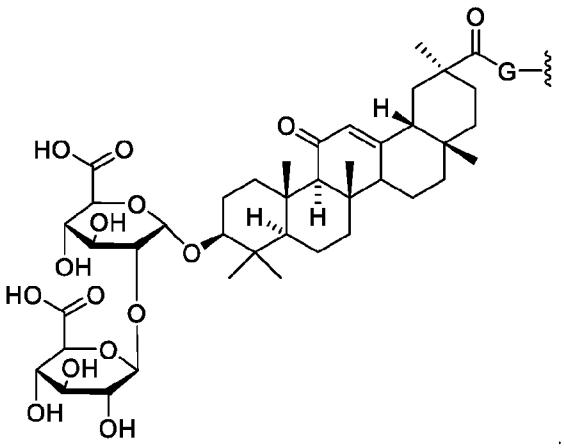
$R^C$ is hydrogen, ($C_1$-$C_8$)alkyl, ($C_1$-$C_8$)haloalkyl, ($C_1$-$C_8$)alkoxy, ($C_1$-$C_6$)alkanoyl, ($C_3$-$C_{20}$)cycloalkyl, ($C_3$-$C_{20}$)heterocycle, aryl, heteroaryl, monosaccharide, disaccharide or trisaccharide; and wherein the cycloalkyl, heterocyle, ary, heteroaryl and saccharide are optionally substituted with one or more groups independently selected from the group consisting of halo, carboxyl, hydroxyl, amino, ($C_1$-$C_4$)alkyl, ($C_1$-$C_4$)haloalkyl, ($C_1$-$C_4$)alkoxy and ($C_1$-$C_4$)haloalkoxy;

or a salt thereof.

In one embodiment $R^C$ is:



In one embodiment $R^1$ is:

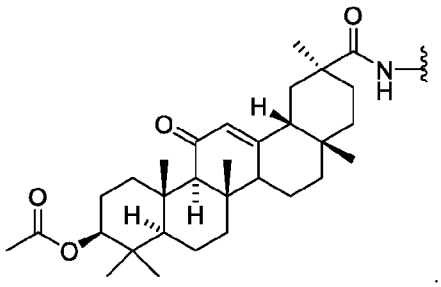


In one embodiment $R^C$ is:

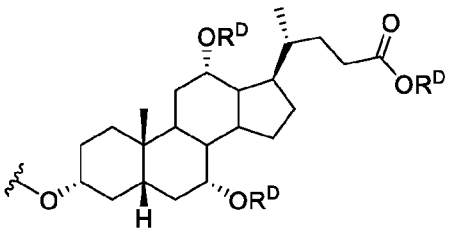


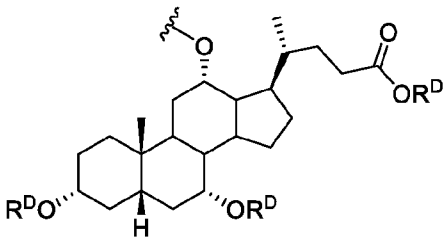
In one embodiment G is –NH-.

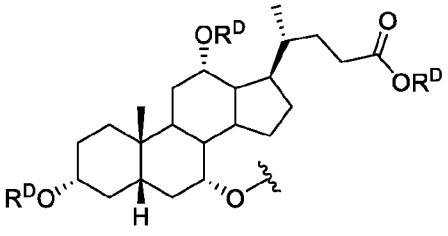
In one embodiment $R^1$ is:



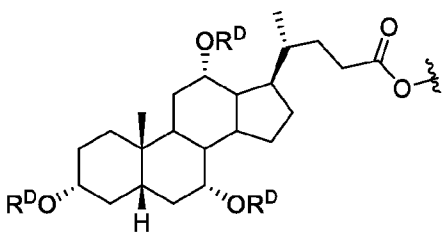
In one embodiment $R^1$ is:







or



wherein each $R^D$ is independently selected from the group consisting of hydrogen, ($C_1$-$C_6$)alkyl, ($C_9$-$C_{20}$)alkylsilyl, $(R^W)_3Si$-, ($C_2$-$C_6$)alkenyl, tetrahydropyranyl, ($C_1$-$C_6$)alkanoyl, benzoyl, aryl($C_1$-$C_3$)alkyl, TMTr (Trimethoxytrityl), DMTr (Dimethoxytrityl), MMTr (Monomethoxytrityl), and Tr (Trityl); and

each $R^W$ is independently selected from the group consisting of ($C_1$-$C_4$)alkyl and aryl.

In one embodiment linking groups $L^1$ and $L^2$ are independently a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or ($C_1$-$C_6$)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkanoyl, ($C_1$-$C_6$)alkanoyloxy, ($C_1$-$C_6$)alkoxycarbonyl, ($C_1$-$C_6$)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

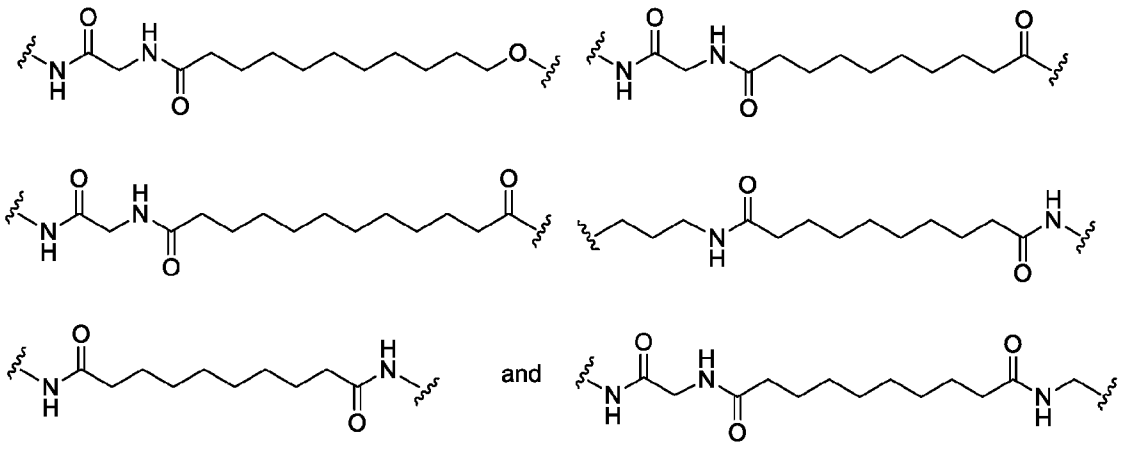
In one embodiment $L^1$ and $L^2$ are independently a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 20 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or ($C_1$-$C_6$)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkanoyl, ($C_1$-$C_6$)alkanoyloxy, ($C_1$-$C_6$)alkoxycarbonyl, ($C_1$-$C_6$)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

In one embodiment $L^1$ and $L^2$ are independently, a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 14 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or ($C_1$-$C_6$)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from ($C_1$-$C_6$)alkoxy, ($C_3$-$C_6$)cycloalkyl, ($C_1$-$C_6$)alkanoyl, ($C_1$-$C_6$)alkanoyloxy, ($C_1$-$C_6$)alkoxycarbonyl, ($C_1$-$C_6$)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

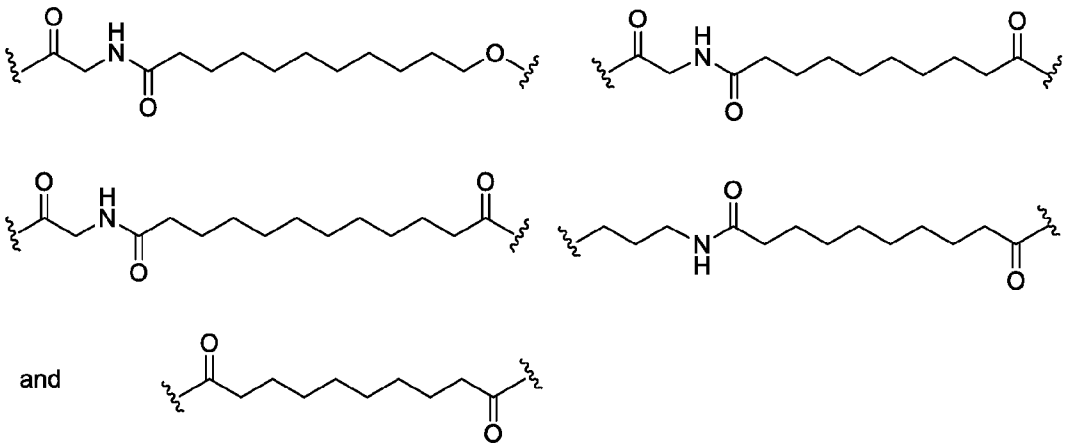
In one embodiment $L^1$ is connected to $R^1$ through -NH-, -O-, -S-, -(C=O)-, -(C=O)-NH-, -NH-(C=O)-, -(C=O)-O-, -NH-(C=O)-NH-, or –NH-($SO_2$)-.

In one embodiment $L^2$ is connected to $R^2$ through -O-.

In one embodiment $L^1$ is selected from the group consisting of:



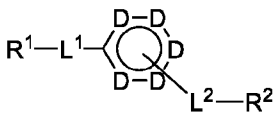

In one embodiment $L^1$ is selected from the group consisting of:




and salts thereof.

In one embodiment $L^2$ is $-CH_2-O-$ or $-CH_2-CH_2-O-$.

In one embodiment a compound of formula I has the following formula Ia:



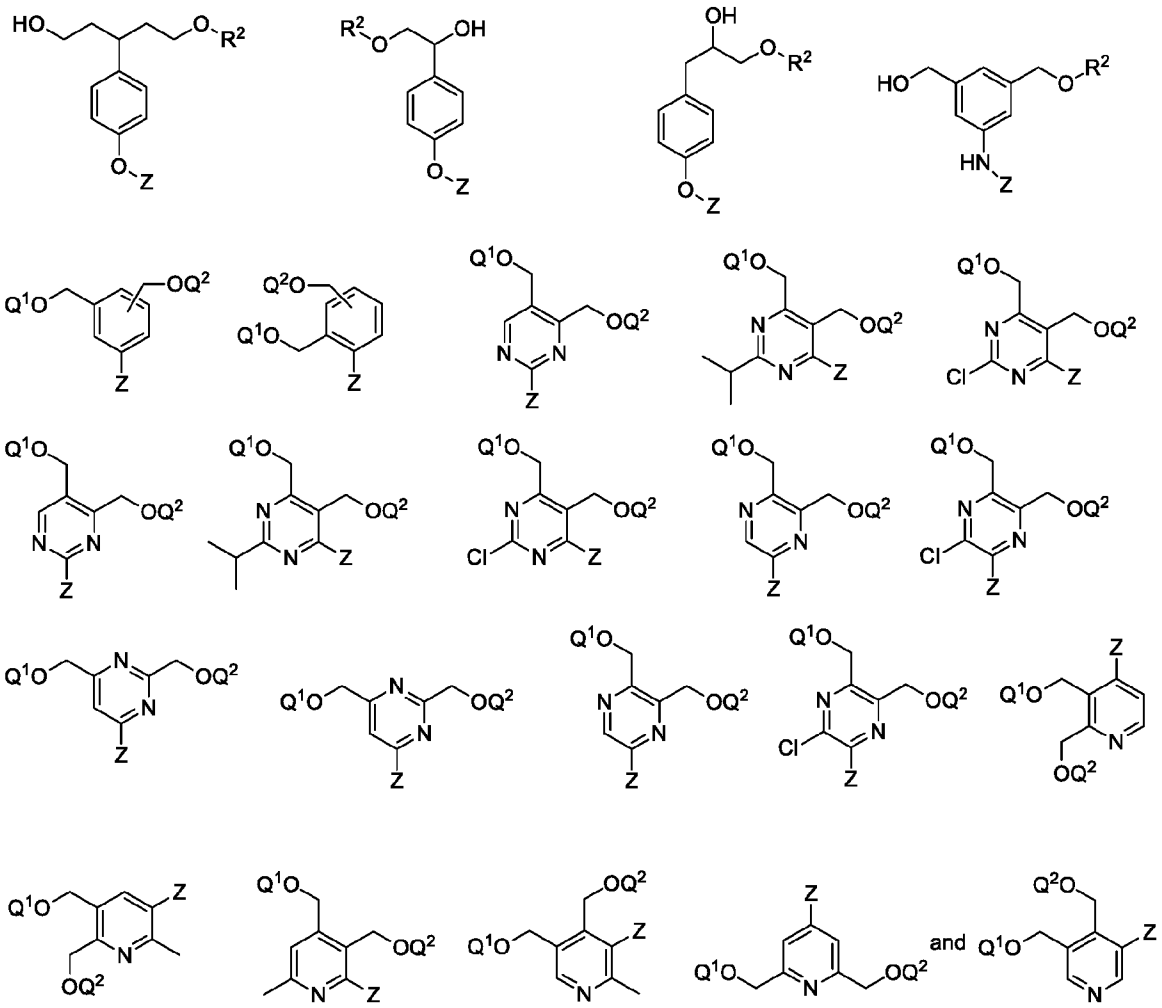

(Ia)

wherein:

each D is independently selected from the group consisting of  and –N=;

or a salt thereof.

In one embodiment a compound of formula Ia is selected from the group consisting of:



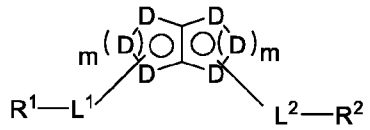
wherein:

$Q^1$ is hydrogen and $Q^2$ is $R^2$; or $Q^1$ is $R^2$ and $Q^2$ is hydrogen;

Z is $–L^1\text{-}R^1$;

and salts thereof.

In one embodiment a compound of formula I has the following formula Ib:



(Ib)

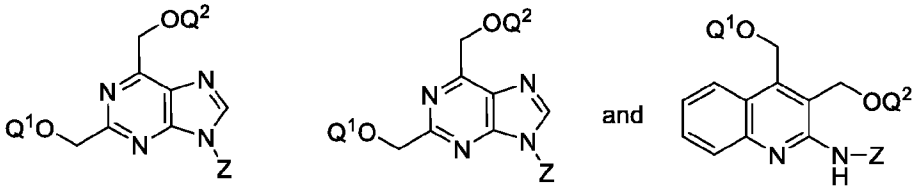
wherein:

each D is independently selected from the group consisting of  and –N=;

each m is independently 1 or 2;

or a salt thereof.

In one embodiment a compound of formula Ib is selected from the group consisting of:



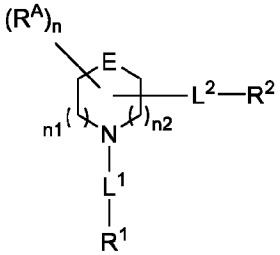
wherein:

$Q^1$ is hydrogen and $Q^2$ is $R^2$; or $Q^1$ is $R^2$ and $Q^2$ is hydrogen;

$Z$ is $-L^1$-$R^1$;

and salts thereof.

In one embodiment a compound of formula I has the following formula (Ic):



(Ic)

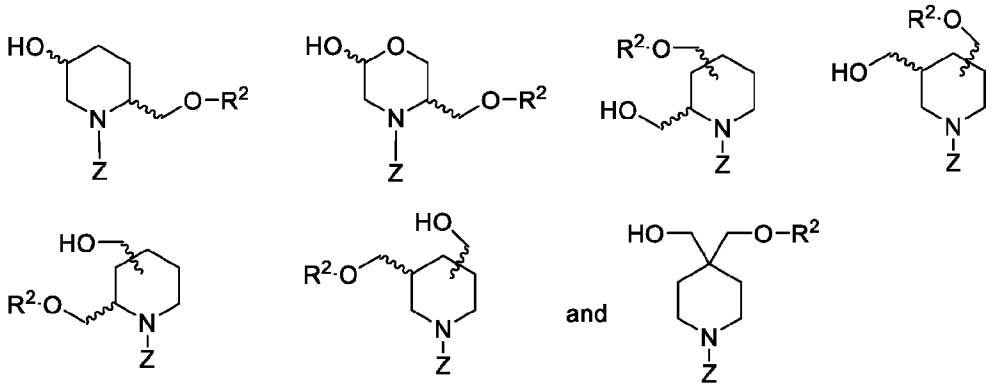
Wherein E is –O- or -$CH_2$-;

n is selected from the group consisting of 0, 1, 2, 3, and 4; and

n1 and n2 are each independently selected from the group consisting of 0, 1, 2, and 3;

or a salt thereof.

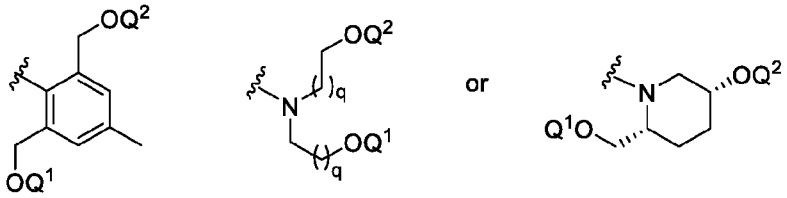
In certain embodiments a compound of formula (Ic) is selected from the group consisting of:



wherein Z is $-L^1$-$R^1$;

and salts thereof.

In one embodiment the -A-$L^2$-$R^2$ moiety is:



wherein:

$Q^1$ is hydrogen and $Q^2$ is $R^2$; or $Q^1$ is $R^2$ and $Q^2$ is hydrogen; and

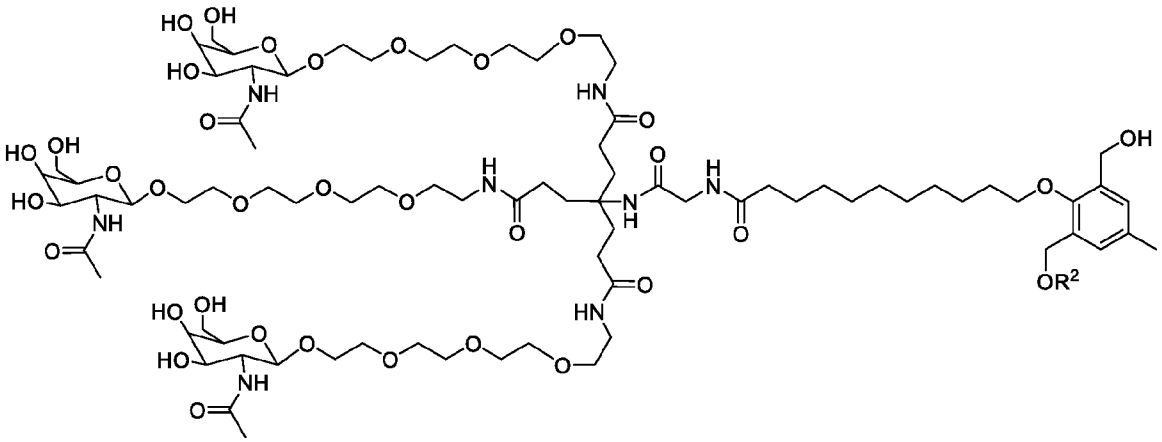
each q is independently 0, 1, 2, 3, 4 or 5;

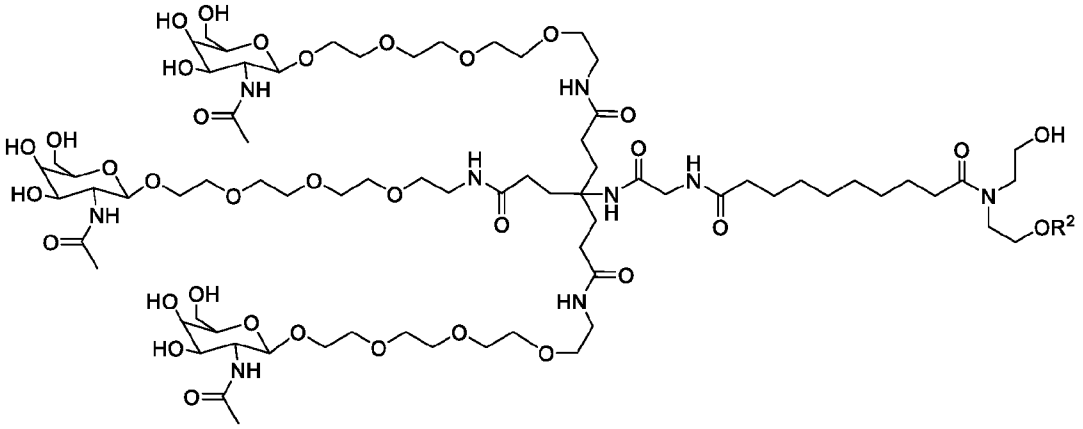
or a salt thereof.

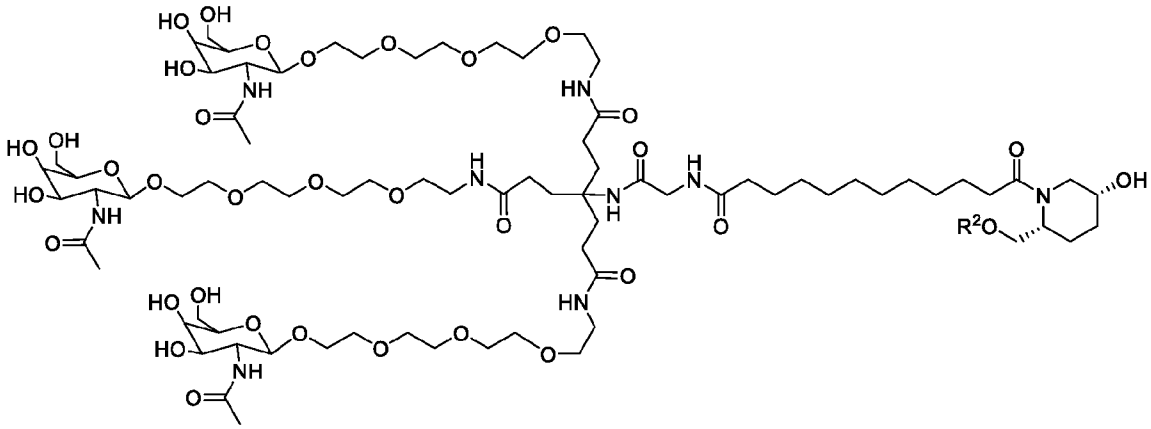
In one embodiment $R^2$ is an oligonucleotide.

In one embodiment $R^2$ is an siRNA.

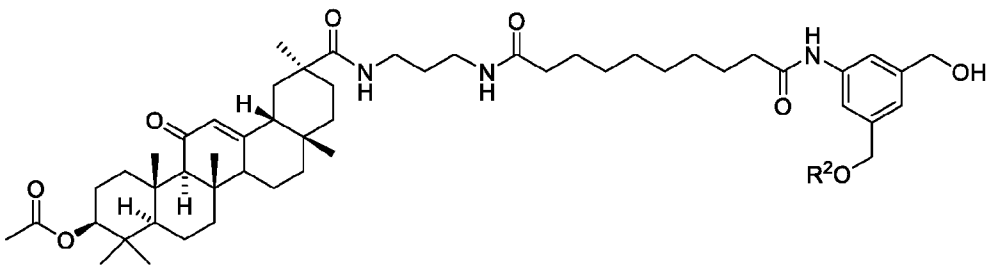
In one embodiment a compound of formula (I) is selected from the group consisting of:





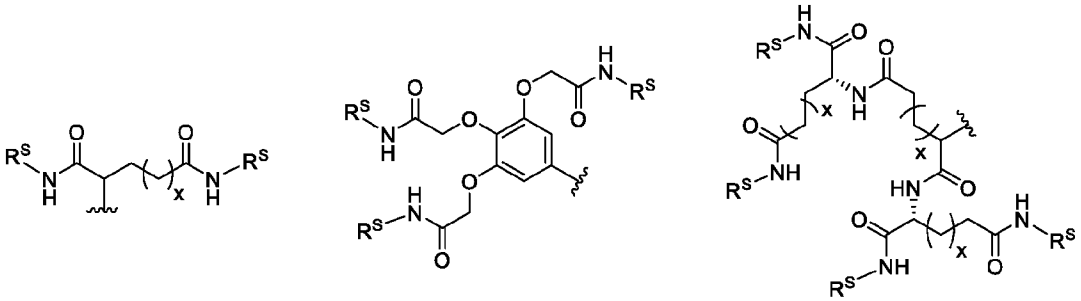


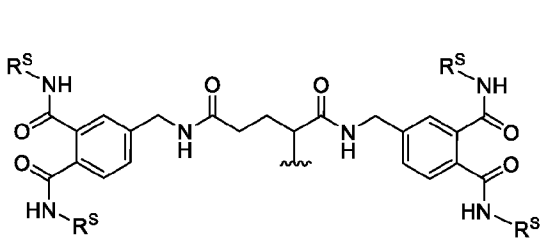
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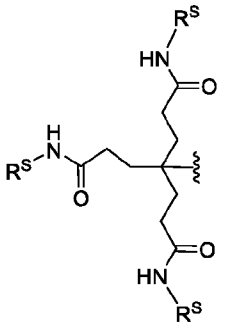


and salts thereof.

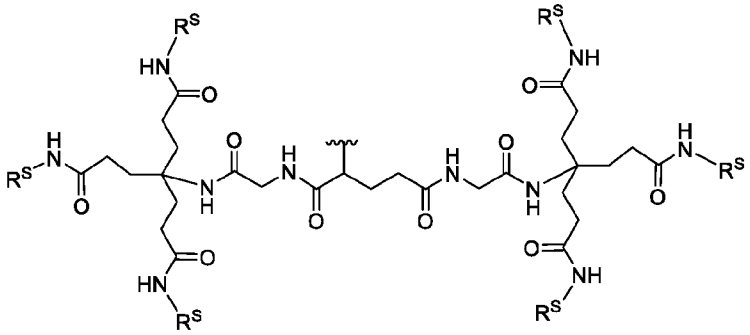
In one embodiment $R^1$ is selected from the group consisting of:

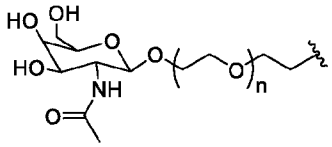






and



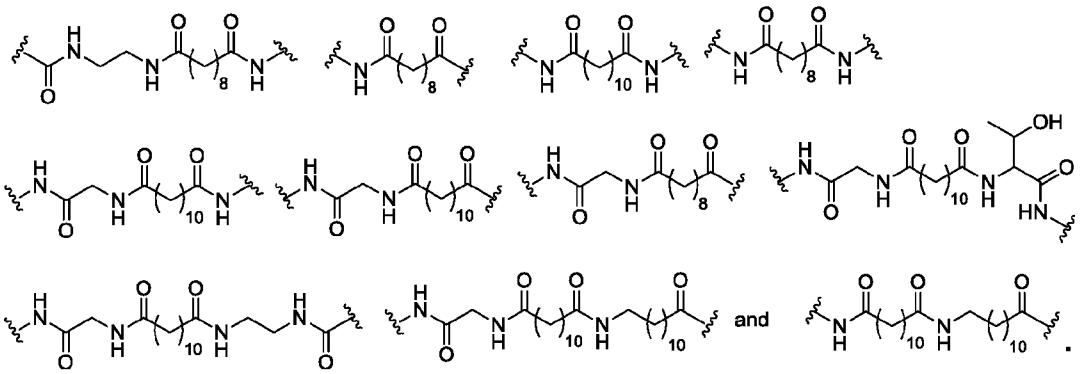


wherein $R^S$ is ;

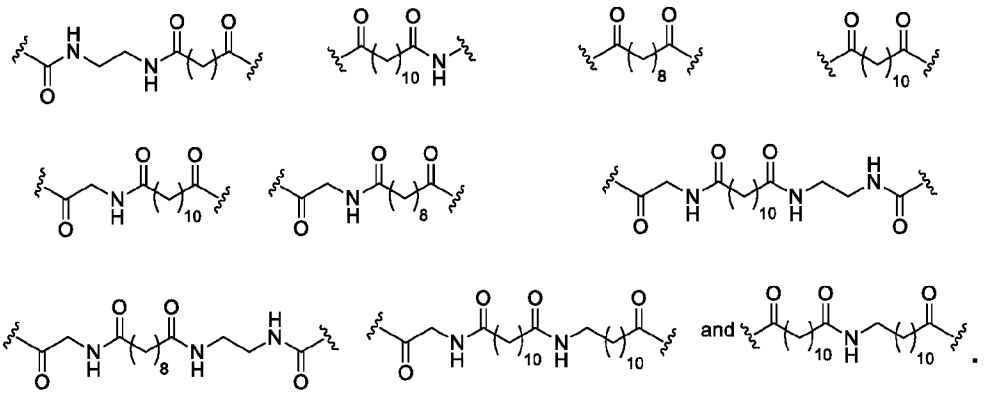
n is 2, 3, or 4;

x is 1 or 2.

In one embodiment $L^1$ is selected from the group consisting of:



In one embodiment $L^1$ is selected from the group consisting of:




In one embodiment A is absent, phenyl, pyrrolidinyl, or cyclopentyl.

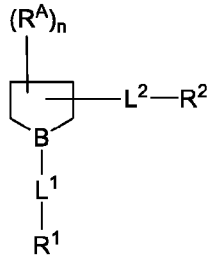
In one embodiment $L^2$ is $C_{1-4}$ alkylene-O- that is optionally substituted with hydroxy.

In one embodiment $L^2$ is $–CH_2O-$, $-CH_2CH_2O-$, or $-CH(OH)CH_2O-$.

In one embodiment each $R^A$ is independently hydroxy or $C_{1-8}$ alkyl that is optionally substituted with hydroxyl.

In one embodiment each $R^A$ is independently selected from the group consisting of hydroxy, methyl and $–CH_2OH$.

In one embodiment a compound of formula I has the following formula (Ig):



(Ig)

wherein B is –N- or -CH-;

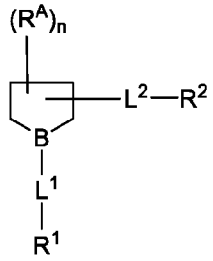
$L^1$ is absent or –NH-;

$L^2$ is $C_{1-4}$ alkylene-O- that is optionally substituted with hydroxyl or halo;

n is 0, 1, or 2;

or a salt thereof.

In one embodiment a compound of formula I has the following formula (Ig):



(Ig)

wherein B is –N- or -CH-;

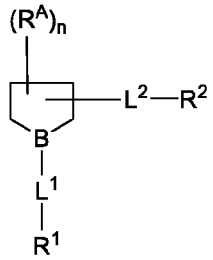
$L^1$ is absent or –NH-;

$L^2$ is $C_{1-4}$ alkylene-O- that is optionally substituted with hydroxyl or halo;

n is 0, 1, 2, 3, 4, 5, 6, or 7;

or a salt thereof.

In one embodiment a compound of formula I has the following formula (Ig):



(Ig)

wherein B is –N- or -CH-;

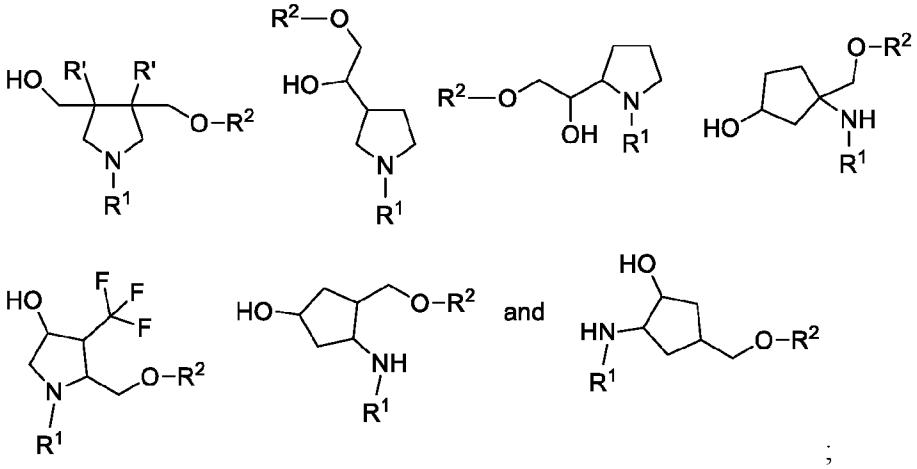
$L^1$ is absent or –NH-;

$L^2$ is $C_{1-4}$ alkylene-O- that is optionally substituted with hydroxyl or halo;

n is 0, 1, 2, 3, or 4;

or a salt thereof.

In one embodiment a compound of formula Ig is selected from the group consisting of:

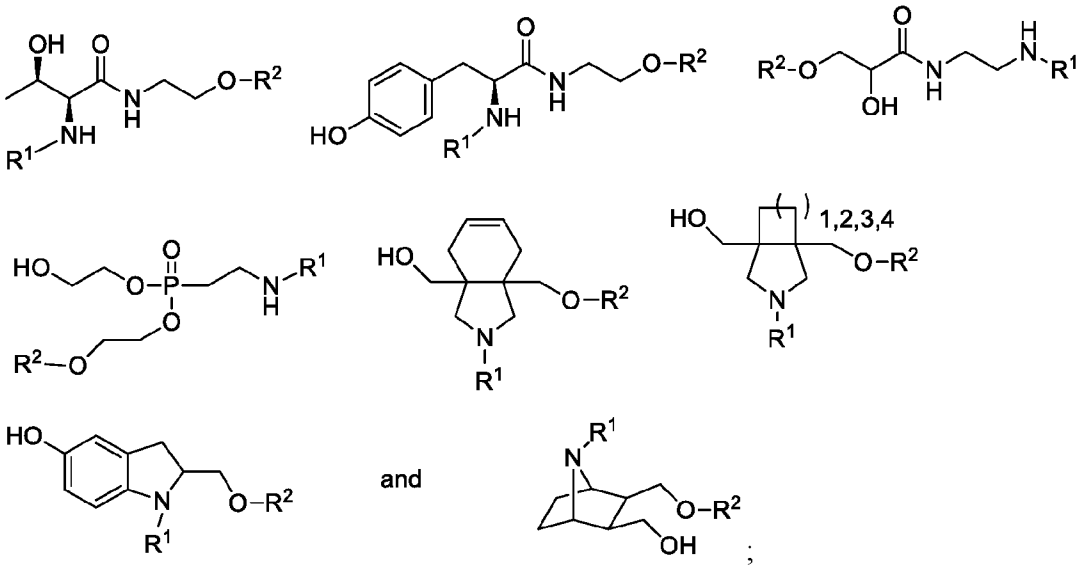


;

wherein R' is $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl; wherein the $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl are optionally substituted with halo or hydroxyl;

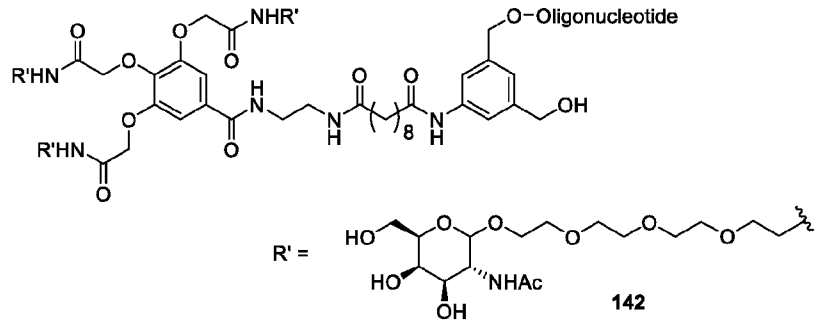
and salts thereof.

In one embodiment a compound of formula I is selected from the group consisting of:

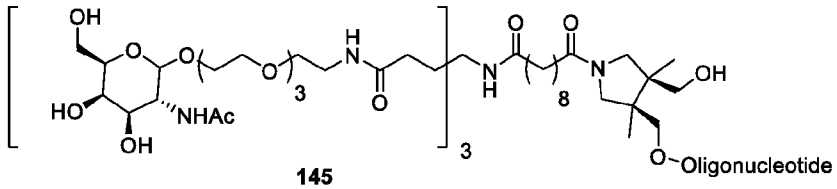


and salts thereof.

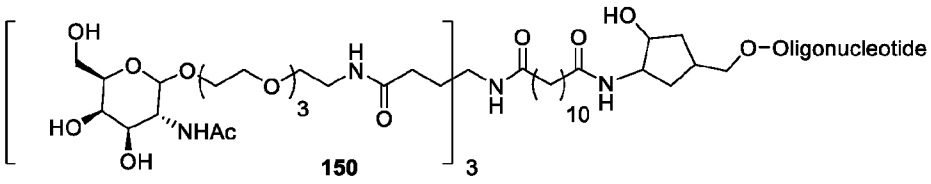
In one embodiment the compound of formula I or the salt thereof is selected from the group consisting of:



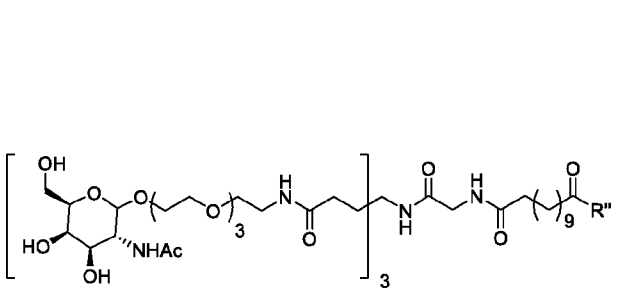
142

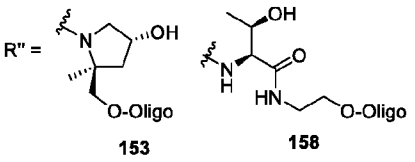


145

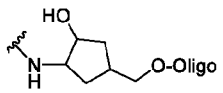


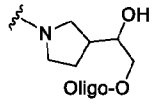
150



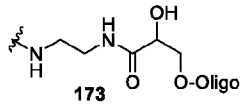


153 158

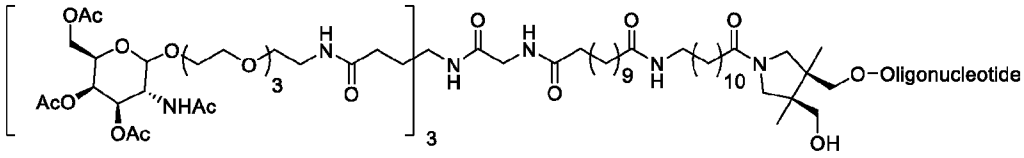




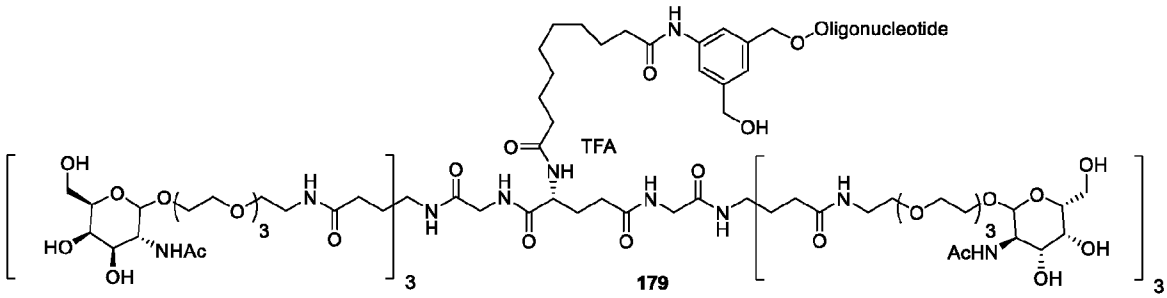
163 168



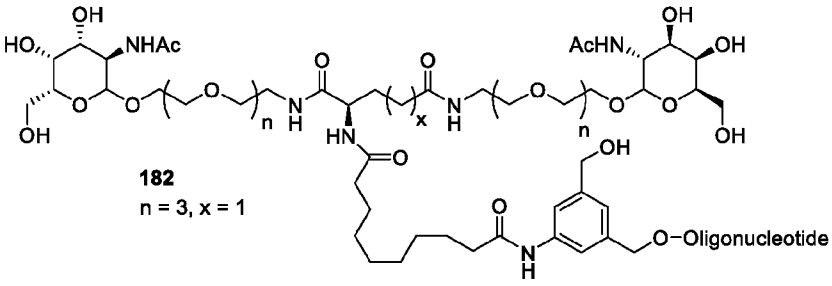
173



176

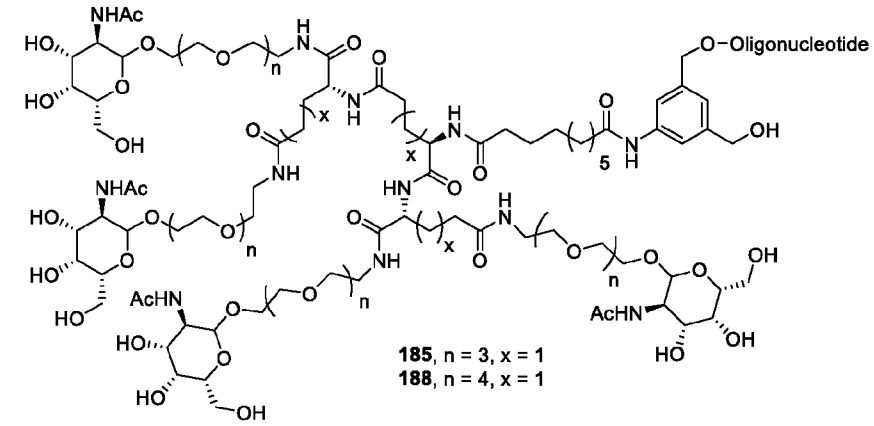


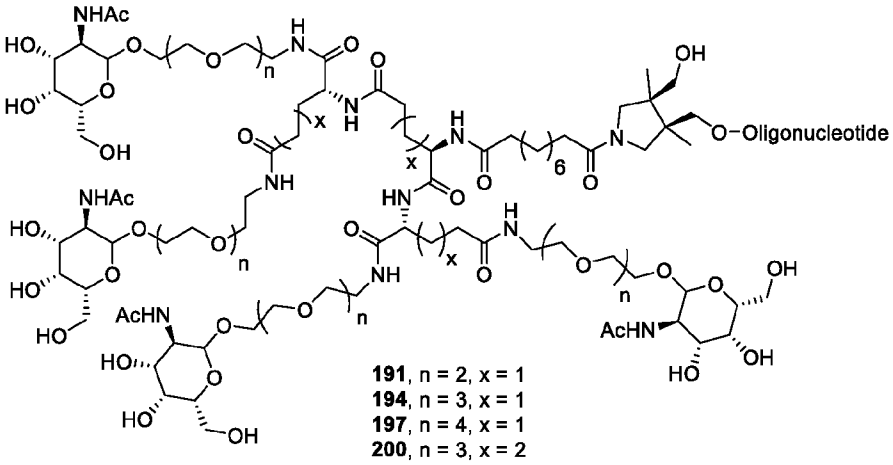
179

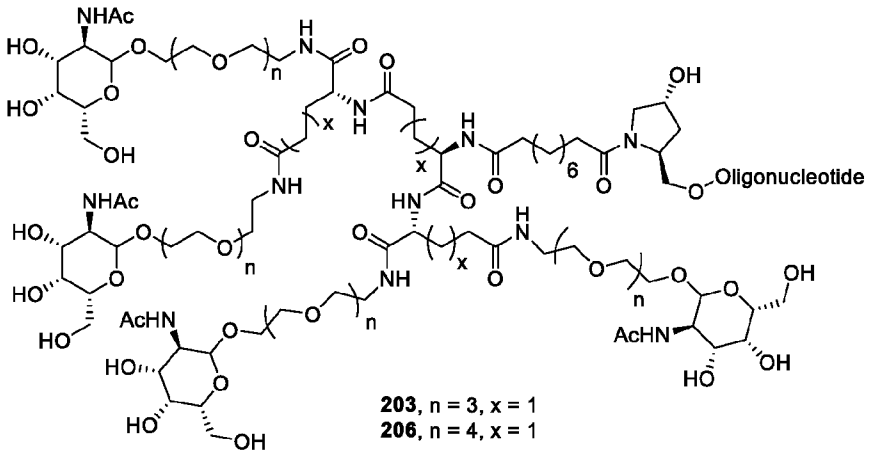


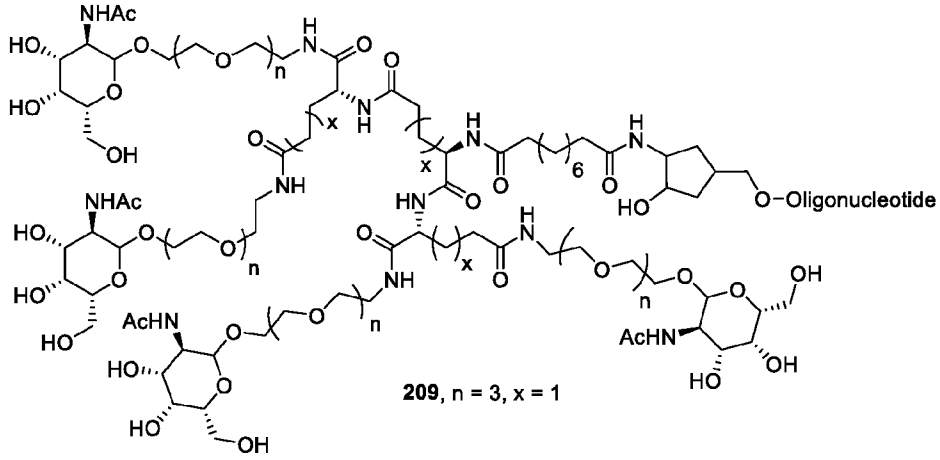
182

n = 3, x = 1

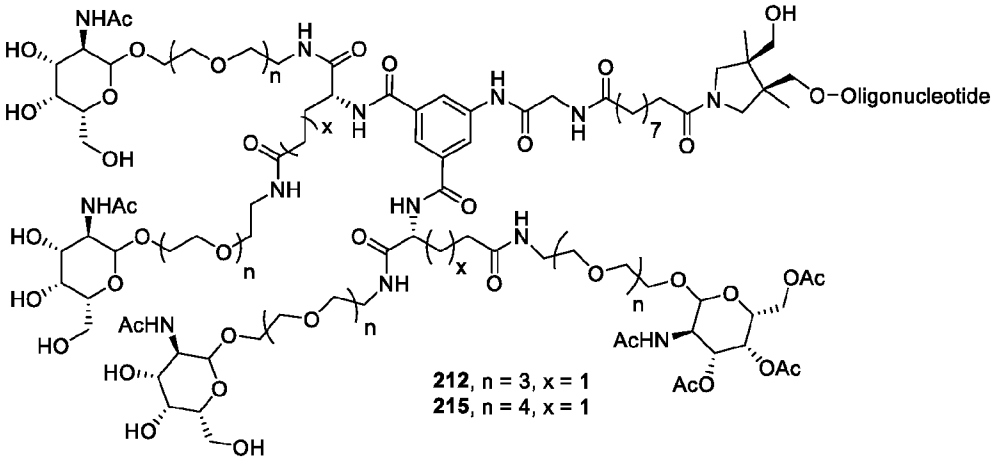

O–Oligonucleotide
185, n = 3, x = 1
188, n = 4, x = 1


O–Oligonucleotide
191, n = 2, x = 1
194, n = 3, x = 1
197, n = 4, x = 1
200, n = 3, x = 2

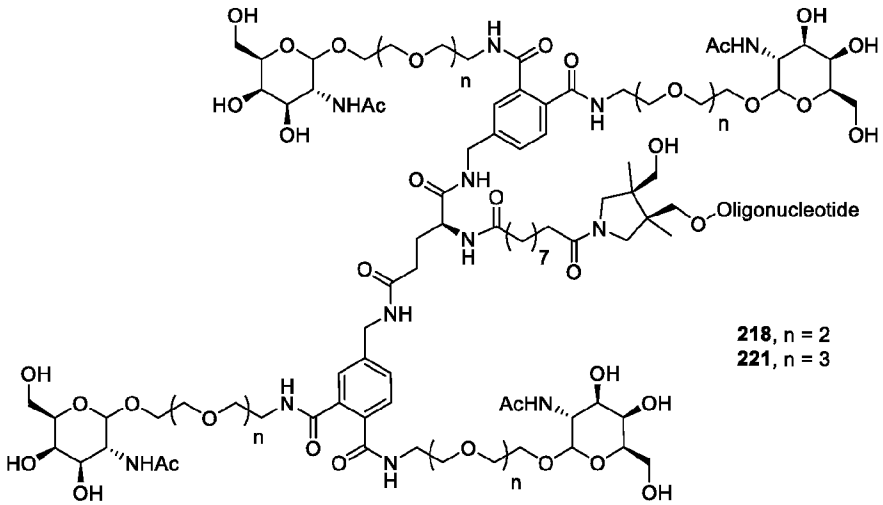

O–Oligonucleotide
203, n = 3, x = 1
206, n = 4, x = 1



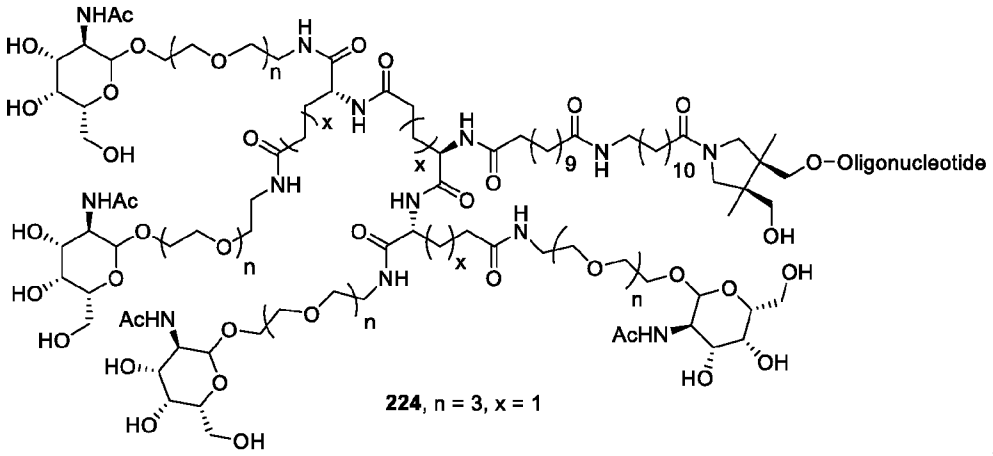

**209**, n = 3, x = 1



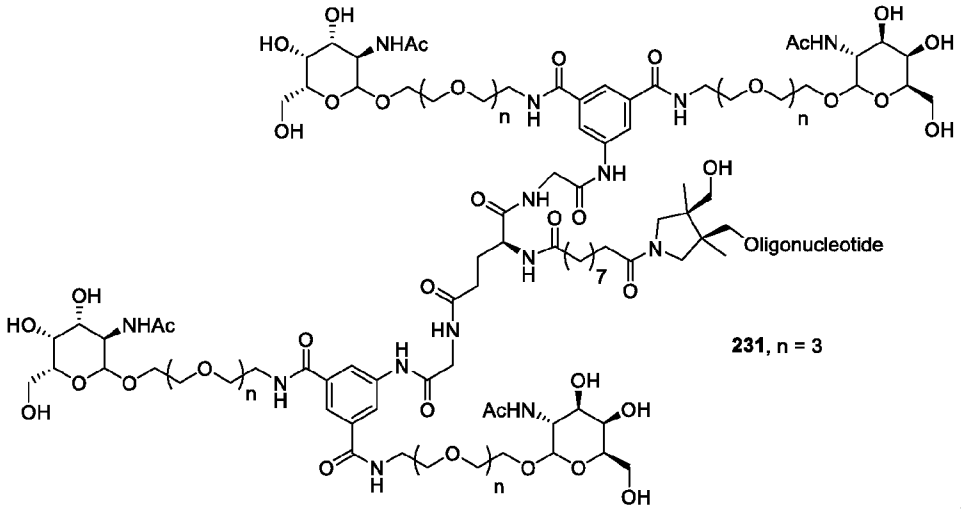

**212**, n = 3, x = 1
**215**, n = 4, x = 1



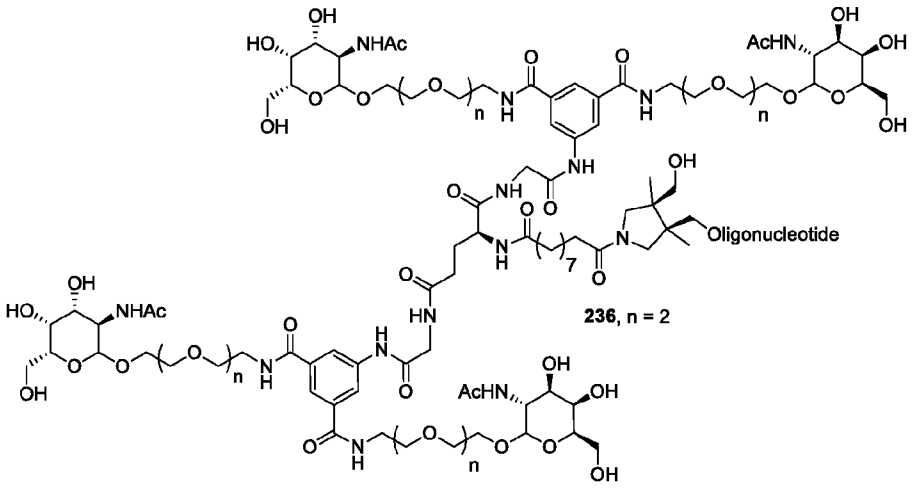

**218**, n = 2
**221**, n = 3




;

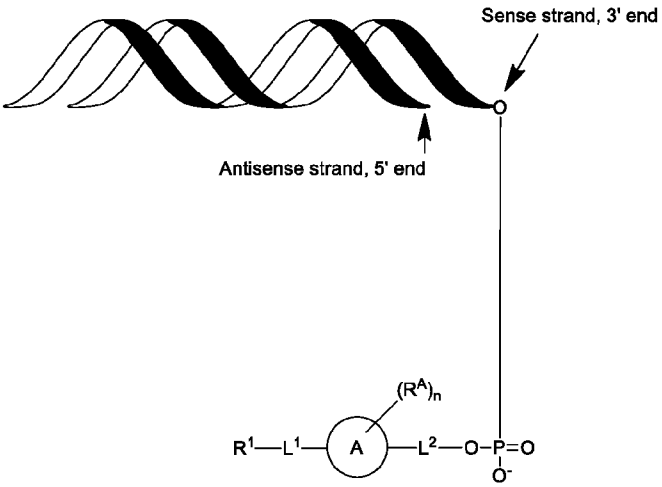



; and




or pharmaceutically acceptable salts thereof.

In certain embodiments, the oligonucleotide is an siRNA molecule. Thus, in one embodiment the compound of formula (I) is,




that is optionally associated with a counter cation.

In one embodiment the conjugate is selected from the group of conjugates shown in the following table, wherein $R^2$ is the modified HBV siRNA shown and is attached through the oxygen of a phosphate at the 3'-end of the sense strand.

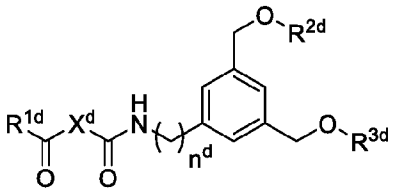
| Formula (I) | siRNA Number | Sense strand SEQ ID NO | Sense strand 5' – 3' | Antisense strand SEQ ID NO | Antisense strand 5'-3' |
|---|---|---|---|---|---|
| | | | | | |
| 145 | 103 | 161 | usgscaCUUcgcuucaccu | 162 | asGsgugaagcgaagUgCacascsgU |
| 145 | 114 | 183 | cscsguguGcACUucgcuuCacc | 184 | gsGsugaAgCgaaguGcAcacGgsuscUU |
| 145 | 120 | 195 | cscsguguGcACUucgcuucaca | 196 | usGsugaAgCGaaguGcAcacggsuscUU |
| 145 | 125 | 205 | gsusgcACUucgcuucaca | 206 | usGsugaagcgaaguGcAcacsgsgU |
| 145 | 134 | 223 | ususuaCuAgUGCcaUuuguuca | 224 | usGsAaCaAauGgcaCuAgUaAascsu |
| 182 | 103 | 161 | usgscaCUUcgcuucaccu | 162 | asGsgugaagcgaagUgCacascsgU |
| 182 | 114 | 183 | cscsguguGcACUucgcuuCacc | 184 | gsGsugaAgCgaaguGcAcacGgsuscUU |
| 182 | 120 | 195 | cscsguguGcACUucgcuucaca | 196 | usGsugaAgCGaaguGcAcacggsuscUU |
| 182 | 125 | 205 | gsusgcACUucgcuucaca | 206 | usGsugaagcgaaguGcAcacsgsgU |
| 182 | 134 | 223 | ususuaCuAgUGCcaUuuguuca | 224 | usGsAaCaAauGgcaCuAgUaAascsu |
| 200 | 103 | 161 | usgscaCUUcgcuucaccu | 162 | asGsgugaagcgaagUgCacascsgU |
| 200 | 114 | 183 | cscsguguGcACUucgcuuCacc | 184 | gsGsugaAgCgaaguGcAcacGgsuscUU |
| 200 | 120 | 195 | cscsguguGcACUucgcuucaca | 196 | usGsugaAgCGaaguGcAcacggsuscUU |
| 200 | 125 | 205 | gsusgcACUucgcuucaca | 206 | usGsugaagcgaaguGcAcacsgsgU |
| 200 | 134 | 223 | ususuaCuAgUGCcaUuuguuca | 224 | usGsAaCaAauGgcaCuAgUaAascsu |
| 215 | 103 | 161 | usgscaCUUcgcuucaccu | 162 | asGsgugaagcgaagUgCacascsgU |
| 215 | 114 | 183 | cscsguguGcACUucgcuuCacc | 184 | gsGsugaAgCgaaguGcAcacGgsuscUU |
| 215 | 120 | 195 | cscsguguGcACUucgcuucaca | 196 | usGsugaAgCGaaguGcAcacggsuscUU |
| 215 | 125 | 205 | gsusgcACUucgcuucaca | 206 | usGsugaagcgaaguGcAcacsgsgU |

| Formula (I) | siRNA Number | Sense strand SEQ ID NO | Sense strand 5' – 3' | Antisense strand SEQ ID NO | Antisense strand 5'-3' |
|---|---|---|---|---|---|
| 215 | 134 | 223 | ususuaCuAgUGCcaUuuguuca | 224 | usGsAaCaAauGgcaCuAgUaAascsu |
| 236 | 103 | 161 | usgscaCUUcgcuucaccu | 162 | asGsgugaagcgaagUgCacascsgU |
| 236 | 114 | 183 | cscsguguGcACUucgcuuCacc | 184 | gsGsugaAgCgaaguGcAcacGgsuscUU |
| 236 | 120 | 195 | cscsguguGcACUucgcuucaca | 196 | usGsugaAgCGaaguGcAcacggsuscUU |
| 236 | 125 | 205 | gsusgcACUucgcuucaca | 206 | usGsugaagcgaaguGcAcacsgsgU |
| 236 | 134 | 223 | ususuaCuAgUGCcaUuuguuca | 224 | usGsAaCaAauGgcaCuAgUaAascsu |

2'-O-Methyl nucleotides = lower case; 2'-Fluoro nucleotides = UPPER CASE;

Phosphorothioate linker = s; Unmodified = UPPER CASE

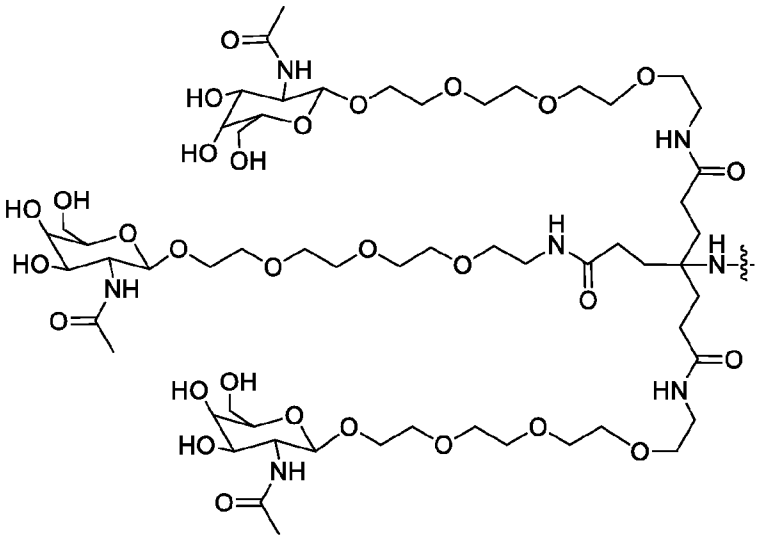
In one embodiment a compound of formula I has the following formula (Id):



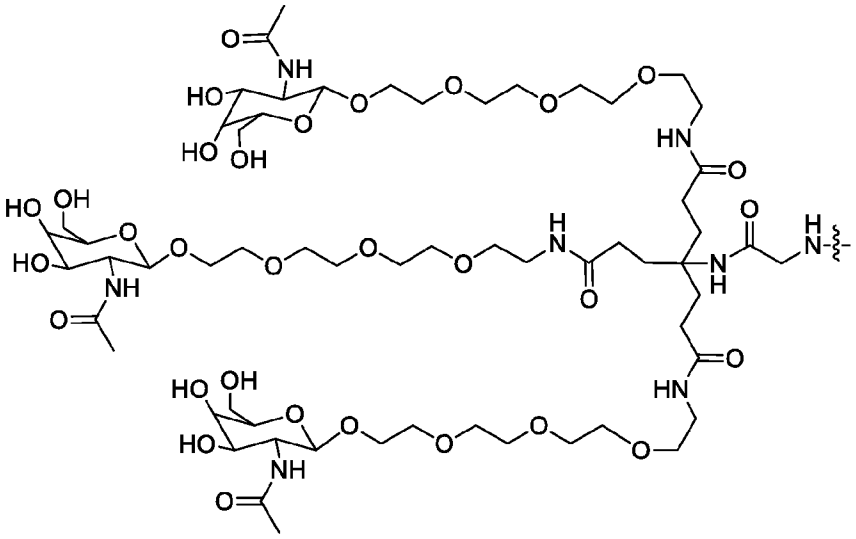
(Id)

wherein:

$R^{1d}$ is selected from:



and



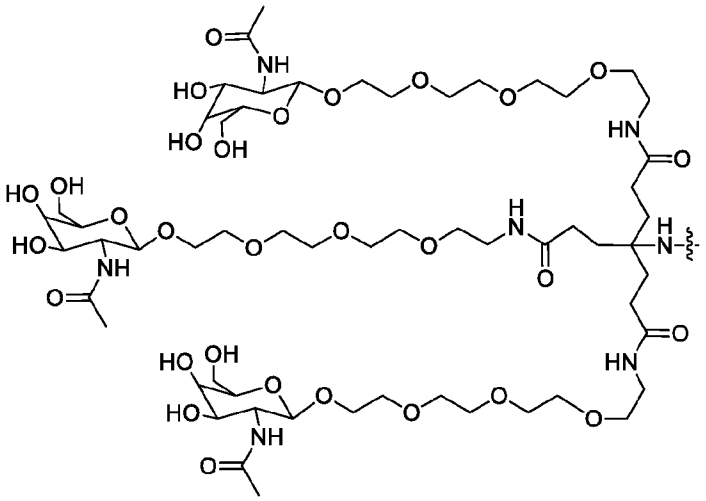
$X^d$ is $C_{2-10}$ alkylene;

$N^d$ is 0 or 1;

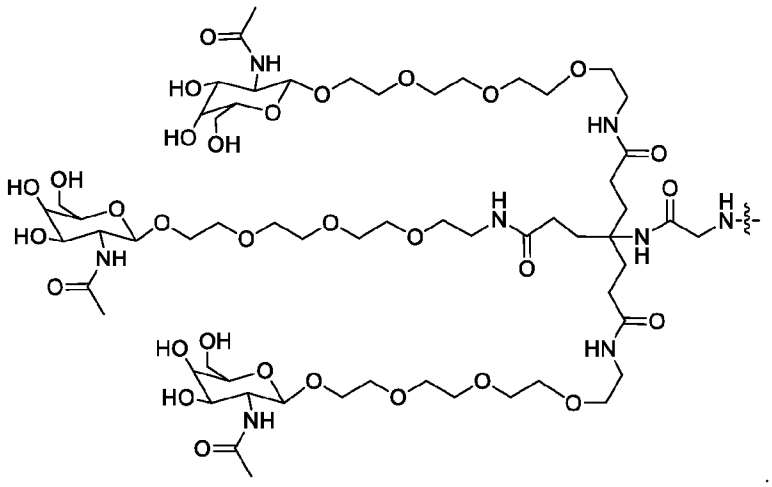
$R^{2d}$ is a nucleic acid; and

$R^{3d}$ is H.

In one embodiment $R^{1d}$ is:



In one embodiment $R^{1d}$ is:

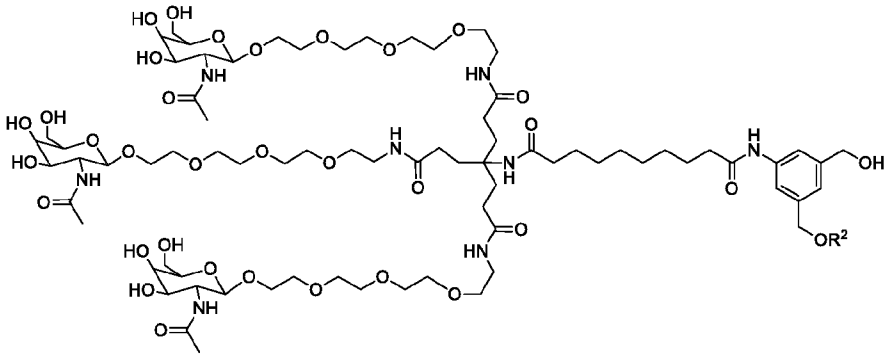


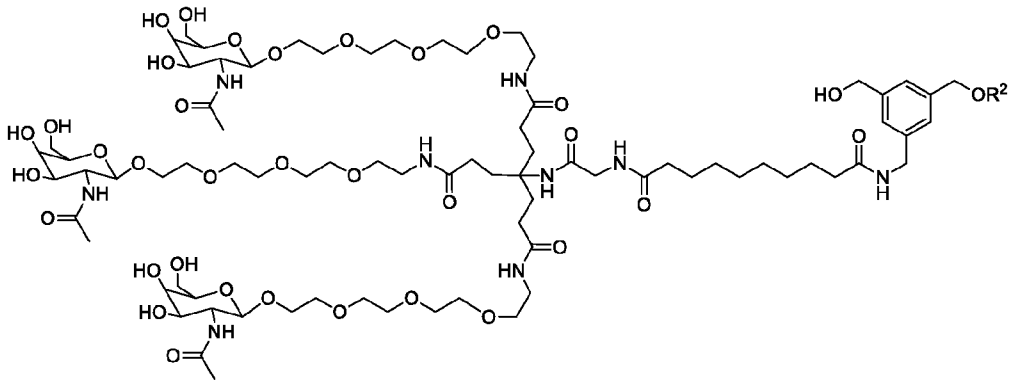
In one embodiment $X^d$ is $C_8$alkylene.

In one embodiment $n^d$ is 0.

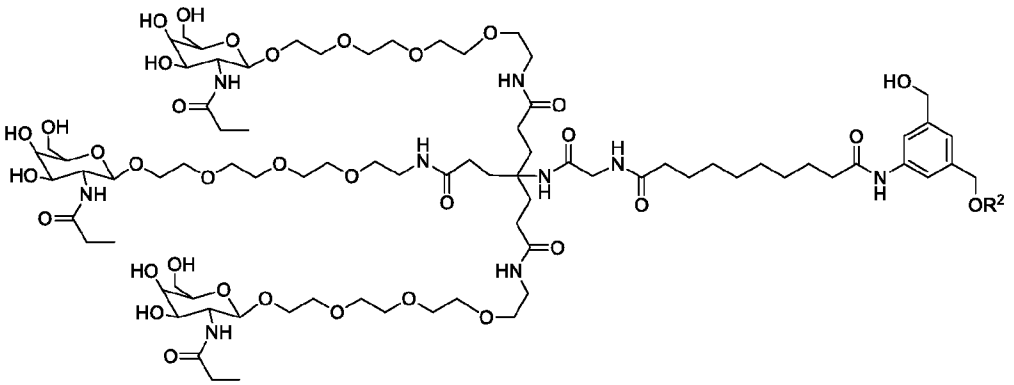
In one embodiment $R^{2d}$ is an siRNA.

In another embodiment a compound of (Id) or the salt thereof is selected from the group consisting of:



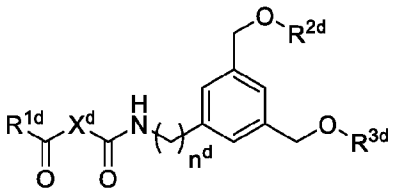


and



and salts thereof.

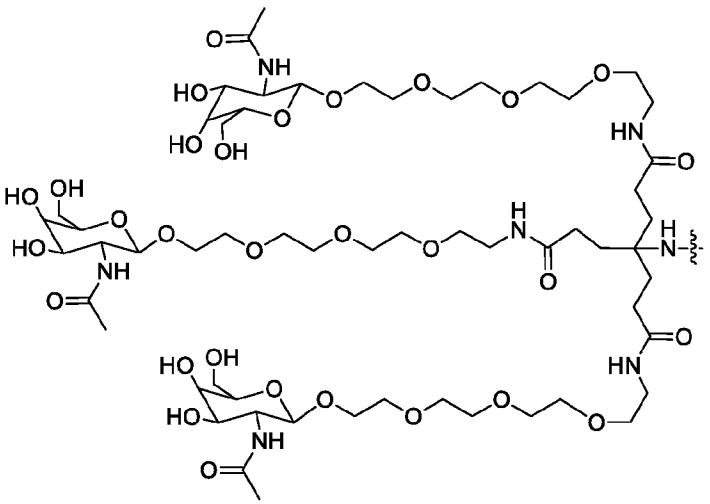
In one embodiment the compound is not a compound formula Id:



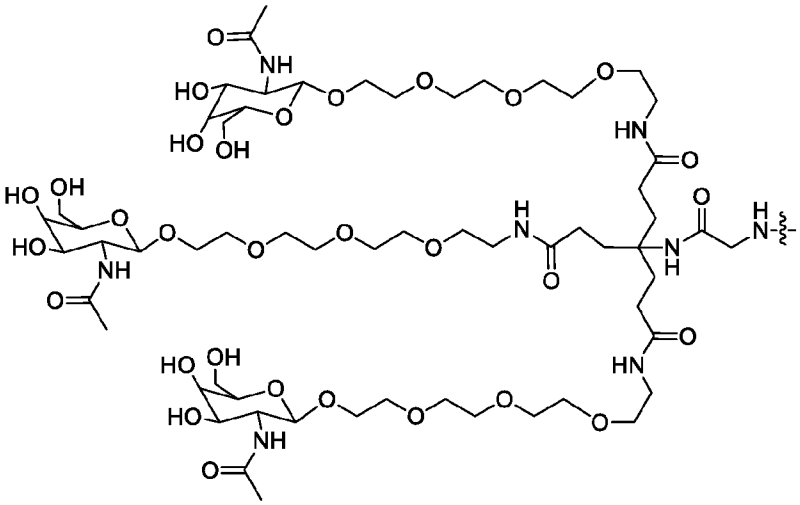
(Id)

or a salt thereof, wherein:

$R^{1d}$ is selected from:



and



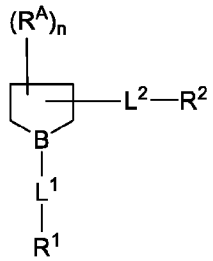
$X^d$ is $C_{2-10}$ alkylene;

$N^d$ is 0 or 1;

$R^{2d}$ is an oligonucleotide; and

$R^{3d}$ is H.

In one embodiment the compound is a compound of formula (Ig):



(Ig)

wherein:

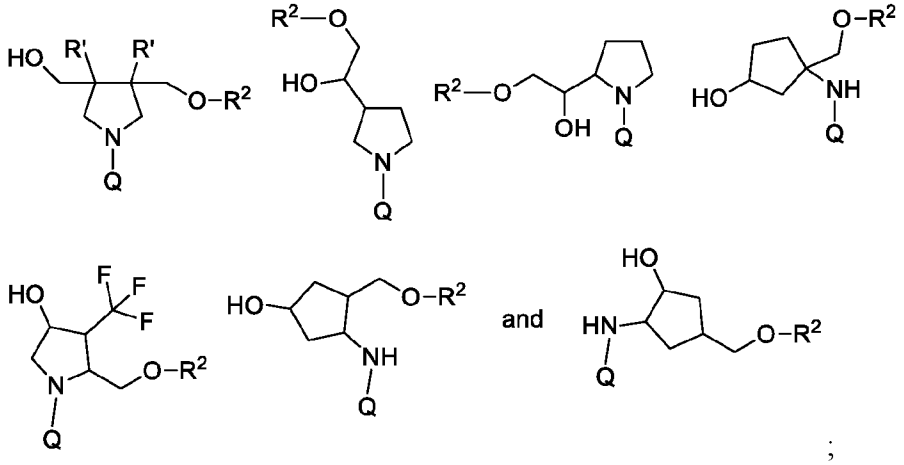
B is –N- or -CH-;

$L^2$ is $C_{1-4}$ alkylene-O- that is optionally substituted with hydroxyl or halo; and

n is 0, 1, 2, 3, 4, 5, 6, or 7;

or a salt thereof.

In one embodiment the compound is a compound selected from the group consisting of:



;

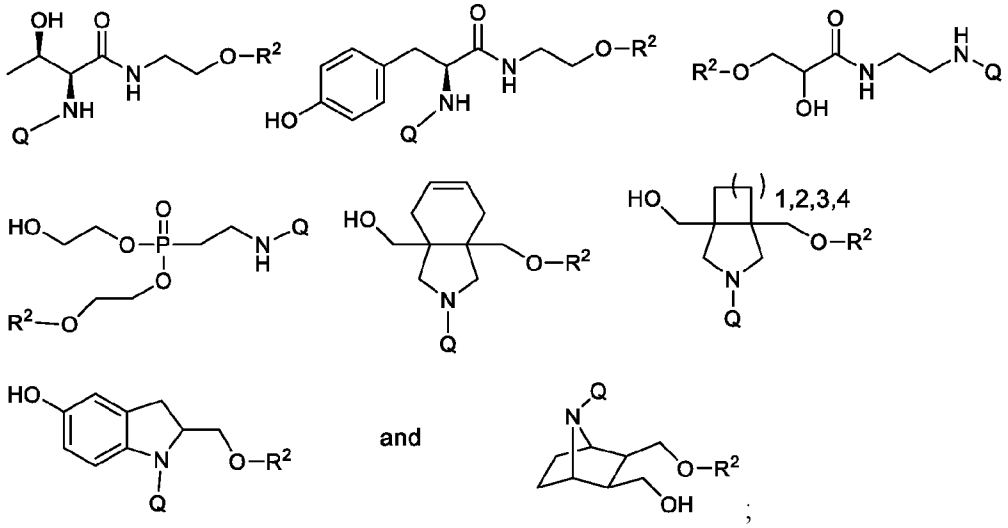
wherein:

Q is $–L^1-R^1$; and

R' is $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl; wherein the $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl are optionally substituted with halo or hydroxyl;

and salts thereof.

In one embodiment the compound is a compound selected from the group consisting of:



;

wherein: Q is $–L^1-R^1$; and salts thereof.

In one embodiment $L^1$ is a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 5 to 20 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of

the carbon atoms in the hydrocarbon chain is optionally replaced –O-, -NH-, -NH-C(=O)-, -C(=O)-NH- or –S-.

In one embodiment the compound is a compound of formula (XX):



(XX)

wherein:

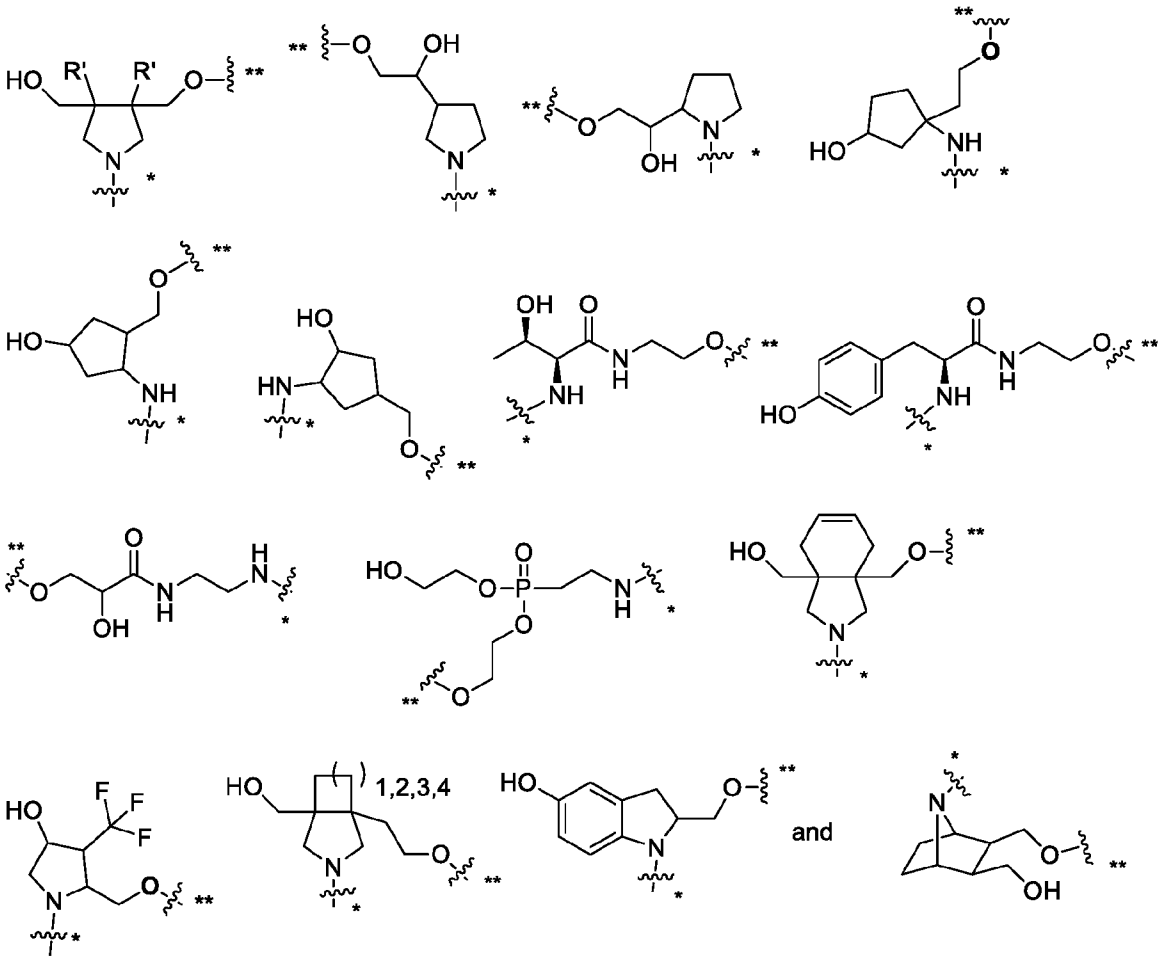
$R^1$ a is targeting ligand;

$L^1$ is absent or a linking group;

$L^2$ is absent or a linking group;

$R^2$ is an oligonucleotide;

B is divalent and is selected from the group consisting of:



wherein:

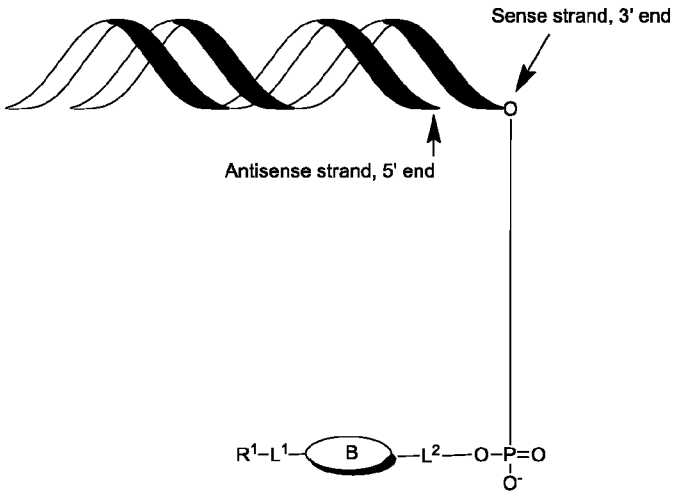
each R' is independently $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl; wherein the $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl are optionally substituted with halo or hydroxyl;

the valence marked with * is attached to $L^1$ or is attached to $R^1$ if $L^1$ is absent; and

the valence marked with ** is attached to $L^2$ or is attached to $R^2$ if $L^2$ is absent;

or a salt thereof.

In one embodiment, the oligonucleotide is an siRNA molecule. Thus, one embodiment of the invention, the compound is a compound of formula (XX),




that is optionally associated with a counter cation.

In one embodiment $R^1$ comprises 2-8 saccharides.

In one embodiment $R^1$ comprises 2-6 saccharides.

In one embodiment $R^1$ comprises 2-4 saccharides.

In one embodiment $R^1$ comprises 3-8 saccharides.

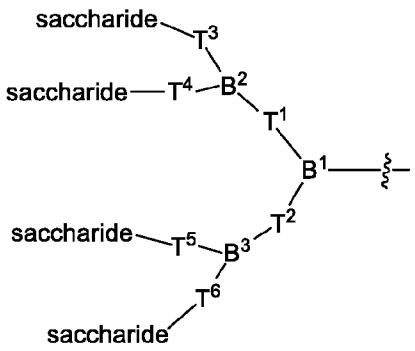
In one embodiment $R^1$ comprises 3-6 saccharides.

In one embodiment $R^1$ comprises 3-4 saccharides.

In one embodiment $R^1$ comprises 3 saccharides.

In one embodiment $R^1$ comprises 4 saccharides.

In one embodiment $R^1$ has the following formula:




wherein:

$B^1$ is a trivalent group comprising about 1 to about 20 atoms and is covalently bonded to $L^1$, $T^1$, and $T^2$.

$B^2$ is a trivalent group comprising about 1 to about 20 atoms and is covalently bonded to $T^1$, $T^3$, and $T^4$;

$B^3$ is a trivalent group comprising about 1 to about 20 atoms and is covalently bonded to $T^2$, $T^5$, and $T^6$;

$T^1$ is absent or a linking group;

$T^2$ is absent or a linking group;

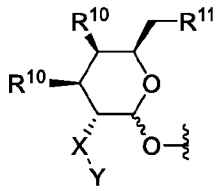
$T^3$ is absent or a linking group;

$T^4$ is absent or a linking group;

$T^5$ is absent or a linking group; and

$T^6$ is absent or a linking group

In one embodiment each saccharide is independently selected from:




wherein:

X is $NR^3$, and Y is selected from $-(C=O)R^4$, $-SO_2R^5$, and $-(C=O)NR^6R^7$; or X is -(C=O)- and Y is $NR^8R^9$;

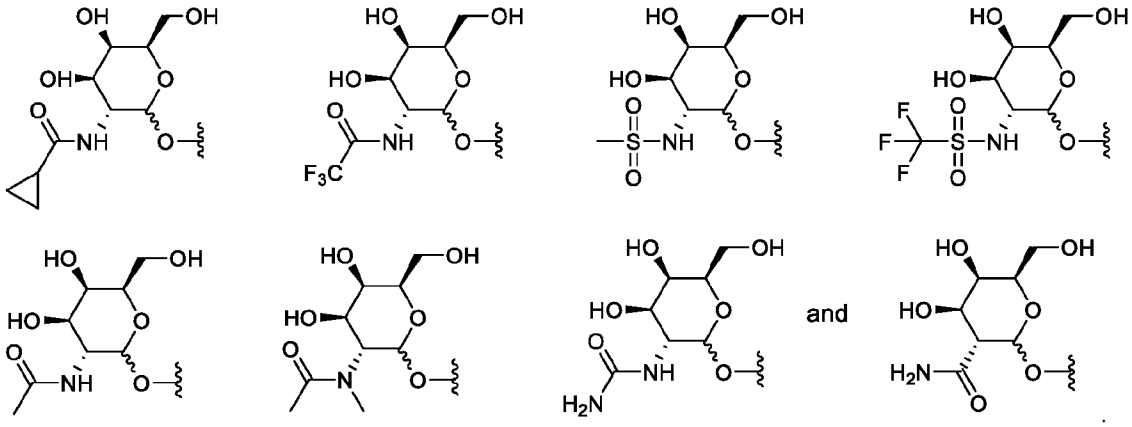
$R^3$ is hydrogen or $(C_1\text{-}C_4)$alkyl;

$R^4$, $R^5$, $R^6$, $R^7$ , $R^8$ and $R^9$ are each independently selected from the group consisting of hydrogen, $(C_1\text{-}C_8)$alkyl, $(C_1\text{-}C_8)$haloalkyl, $(C_1\text{-}C_8)$alkoxy and $(C_3\text{-}C_6)$cycloalkyl that is optionally substituted with one or more groups independently selected from the group consisting of halo, $(C_1\text{-}C_4)$alkyl, $(C_1\text{-}C_4)$haloalkyl, $(C_1\text{-}C_4)$alkoxy and $(C_1\text{-}C_4)$haloalkoxy;

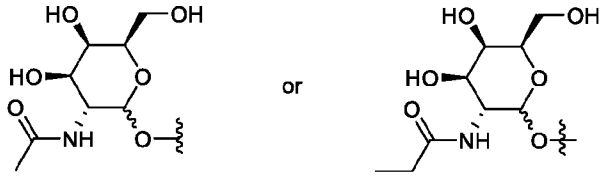
$R^{10}$ is -OH, $-NR^8R^9$ or – F; and

$R^{11}$ is -OH, $-NR^8R^9$, -F or 5 membered heterocycle that is optionally substituted with one or more groups independently selected from the group consisting of halo, hydroxyl, carboxyl, amino, $(C_1\text{-}C_4)$alkyl, $(C_1\text{-}C_4)$haloalkyl, $(C_1\text{-}C_4)$alkoxy and $(C_1\text{-}C_4)$haloalkoxy.

In one embodiment each saccharide is independently selected from the group consisting of:




In one embodiment each saccharide is independently:




In one embodiment one of $T^1$ and $T^2$ is absent.

In one embodiment both $T^1$ and $T^2$ are absent.

In one embodiment each of $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ is independently absent or a branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or (C1-C6)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from (C1-C6)alkoxy, (C3-C6)cycloalkyl, (C1-C6)alkanoyl, (C1-C6)alkanoyloxy, (C1-C6)alkoxycarbonyl, (C1-C6)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

In one embodiment each of $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ is independently absent or a branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 20 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or (C1-C6)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from (C1-C6)alkoxy, (C3-C6)cycloalkyl, (C1-C6)alkanoyl, (C1-C6)alkanoyloxy, (C1-C6)alkoxycarbonyl, (C1-

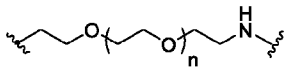
C6)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

In one embodiment each of $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ is independently absent or a branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, or a salt thereof, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O- or -$NR^X$-, and wherein $R^X$ is hydrogen or ($C_1$-$C_6$)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from halo, hydroxy, and oxo (=O).

In one embodiment each of $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ is independently absent or a branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 20 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O- and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from halo, hydroxy, and oxo (=O).

In one embodiment each of $T^1$, $T^2$, $T^3$, $T^4$, $T^5$, and $T^6$ is independently absent or a branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 20 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O- and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from halo, hydroxy, and oxo (=O).

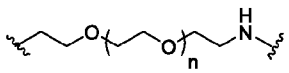
In one embodiment at least one of $T^3$, $T^4$, $T^5$, and $T^6$ is:



wherein:

n = 1, 2, 3.

In one embodiment each of $T^3$, $T^4$, $T^5$, and $T^6$ is independently selected from the group consisting of:



wherein:

n = 1, 2, 3.

In one embodiment at least one of $T^1$ and $T^2$ is glycine

In one embodiment each of $T^1$ and $T^2$ is glycine.

In one embodiment $B^1$ is a trivalent group comprising 1 to 15 atoms and is covalently bonded to $L^1$, $T^1$, and $T^2$.

In one embodiment $B^1$ is a trivalent group comprising 1 to 10 atoms and is covalently bonded to $L^1$, $T^1$, and $T^2$.

In one embodiment $B^1$ comprises a $(C_1\text{-}C_6)$alkyl.

In one embodiment $B^1$ comprises a $C_{3\text{-}8}$ cycloalkyl.

In one embodiment $B^1$ comprises a silyl group.

In one embodiment $B^1$ comprises a D- or L-amino acid.

In one embodiment $B^1$ comprises a saccharide.

In one embodiment $B^1$ comprises a phosphate group.

In one embodiment $B^1$ comprises a phosphonate group.

In one embodiment $B^1$ comprises an aryl.

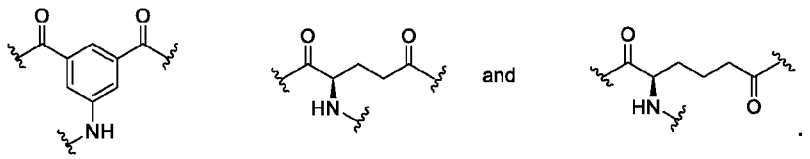
In one embodiment $B^1$ comprises a phenyl ring.

In one embodiment $B^1$ is a phenyl ring.

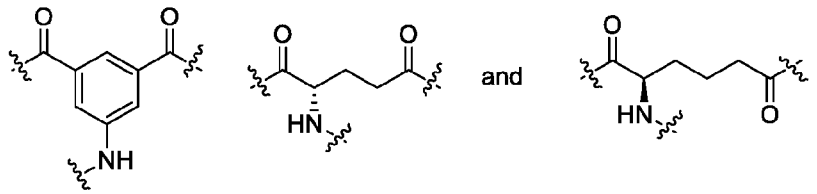
In one embodiment $B^1$ is CH.

In one embodiment $B^1$ comprises a heteroaryl.

In one embodiment $B^1$ is selected from the group consisting of:




In one embodiment $B^1$ is selected from the group consisting of:




In one embodiment $B^2$ is a trivalent group comprising 1 to 15 atoms and is covalently bonded to $L^1$, $T^1$, and $T^2$.

In one embodiment $B^2$ is a trivalent group comprising 1 to 10 atoms and is covalently bonded to $L^1$, $T^1$, and $T^2$.

In one embodiment $B^2$ comprises a $(C_1\text{-}C_6)$alkyl

In one embodiment $B^2$ comprises a $C_{3\text{-}8}$ cycloalkyl.

In one embodiment $B^2$ comprises a silyl group.

In one embodiment $B^2$ comprises a D- or L-amino acid.

In one embodiment $B^2$ comprises a saccharide.

In one embodiment $B^2$ comprises a phosphate group.

In one embodiment $B^2$ comprises a phosphonate group.

In one embodiment $B^2$ comprises an aryl.

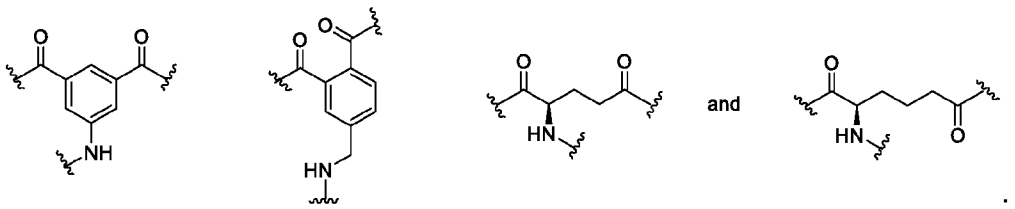
In one embodiment $B^2$ comprises a phenyl ring.

In one embodiment $B^2$ is a phenyl ring.

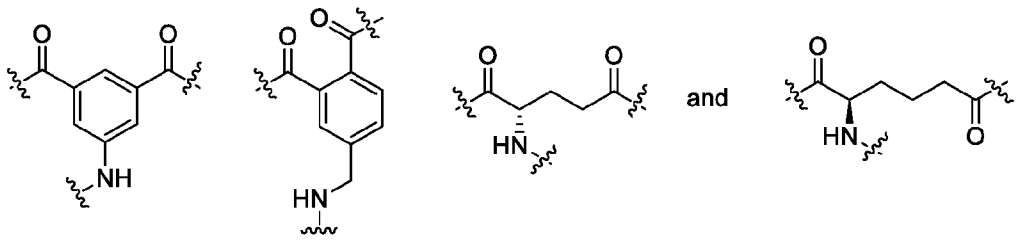
In one embodiment $B^2$ is CH.

In one embodiment $B^2$ comprises a heteroaryl.

In one embodiment $B^2$ is selected from the group consisting of:




In one embodiment $B^2$ is selected from the group consisting of:




In one embodiment $B^3$ is a trivalent group comprising 1 to 15 atoms and is covalently bonded to $L^1$, $T^1$, and $T^2$.

In one embodiment $B^3$ is a trivalent group comprising 1 to 10 atoms and is covalently bonded to $L^1$, $T^1$, and $T^2$.

In one embodiment $B^3$ comprises a $(C_1\text{-}C_6)$alkyl.

In one embodiment $B^3$ comprises a $C_{3\text{-}8}$ cycloalkyl.

In one embodiment $B^3$ comprises a silyl group.

In one embodiment $B^3$ comprises a D- or L-amino acid.

In one embodiment $B^3$ comprises a saccharide.

In one embodiment $B^3$ comprises a phosphate group.

In one embodiment $B^3$ comprises a phosphonate group.

In one embodiment $B^3$ comprises an aryl.

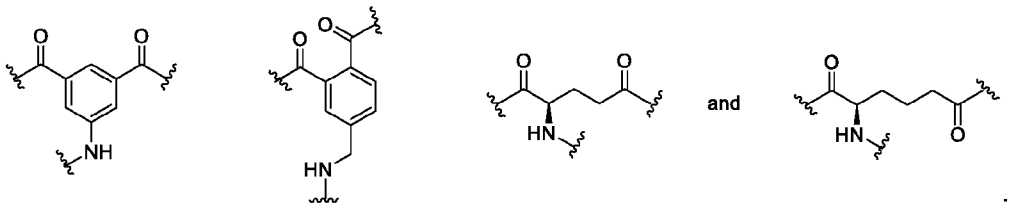
In one embodiment $B^3$ comprises a phenyl ring.

In one embodiment $B^3$ is a phenyl ring.

In one embodiment $B^3$ is CH.

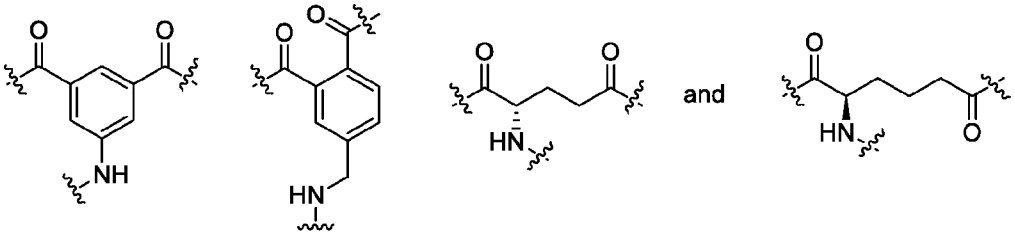
In one embodiment $B^3$ comprises a heteroaryl.

In one embodiment $B^3$ is selected from the group consisting of:



or a salt thereof.

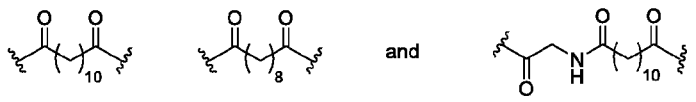
In one embodiment $B^3$ is selected from the group consisting of:



or a salt thereof.

In one embodiment $L^1$ and $L^2$ are independently a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, $-NR^X-$, $-NR^X-C(=O)-$, $-C(=O)-NR^X-$ or –S-, and wherein $R^X$ is hydrogen or (C1-C6)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more (e.g. 1, 2, 3, or 4) substituents selected from (C1-C6)alkoxy, (C3-C6)cycloalkyl, (C1-C6)alkanoyl, (C1-C6)alkanoyloxy, (C1-C6)alkoxycarbonyl, (C1-C6)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

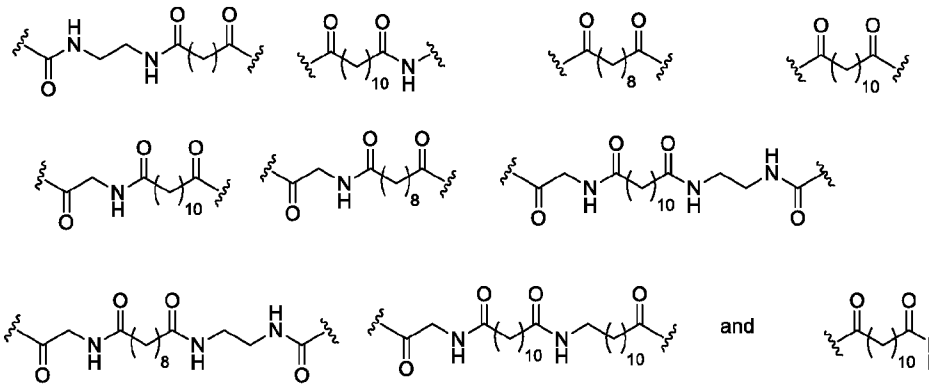
In one embodiment $L^1$ is selected from the group consisting of:



or a salt thereof.

In one embodiment $L^1$ is connected to $B^1$ through a linkage selected from the group consisting of: -O-, -S-, -(C=O)-, -(C=O)-NH-, -NH-(C=O), -(C=O)-O-, -NH-(C=O)-NH-, or –NH-($SO_2$)-.

In one embodiment $L^1$ is selected from the group consisting of:



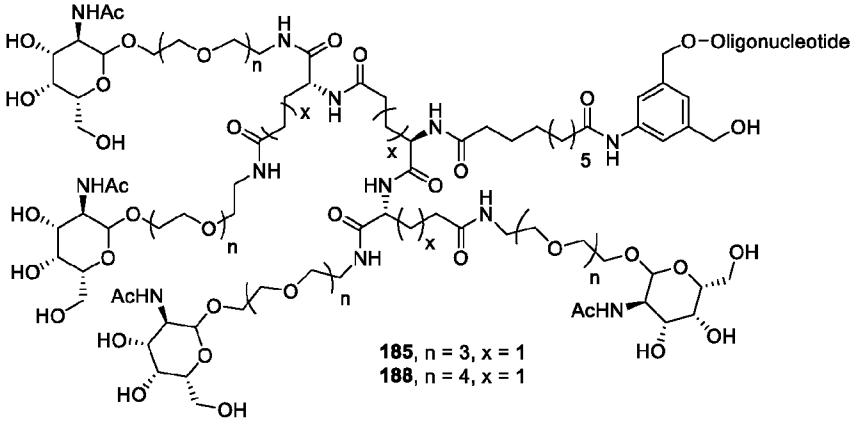
In one embodiment $L^2$ is connected to $R^2$ through -O-.

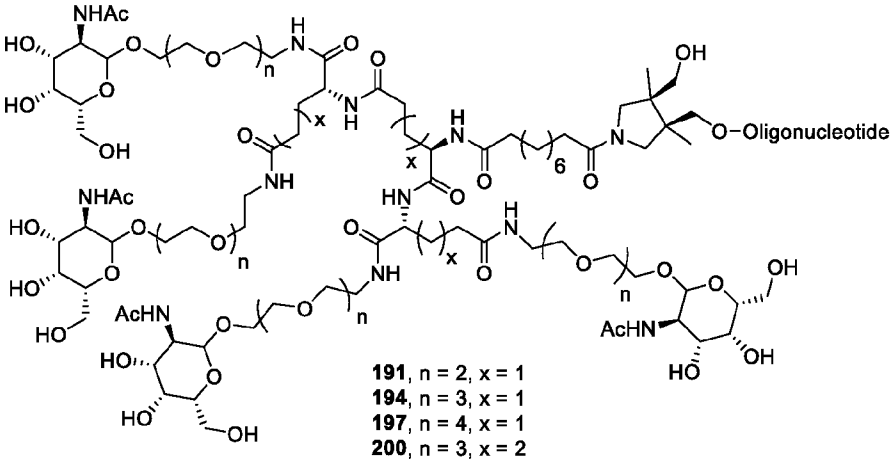
In one embodiment $L^2$ is $C_{1-4}$ alkylene-O- that is optionally substituted with hydroxy.

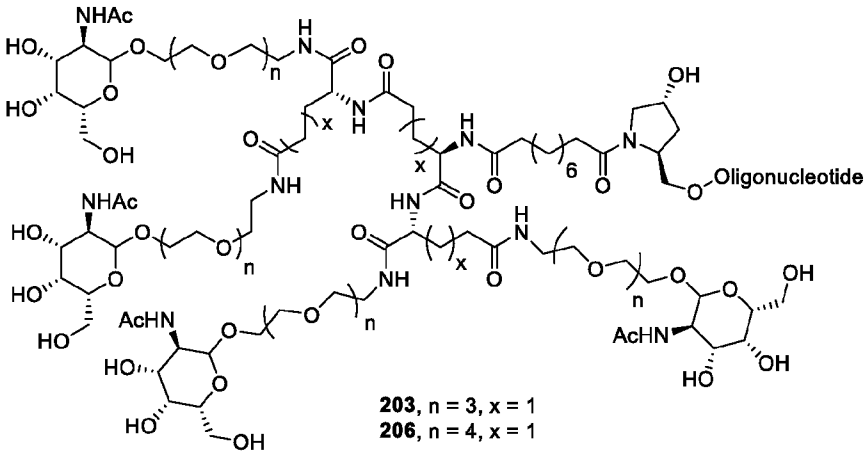
In one embodiment $L^2$ is connected to $R^2$ through -O-.

In one embodiment $L^2$ is absent.

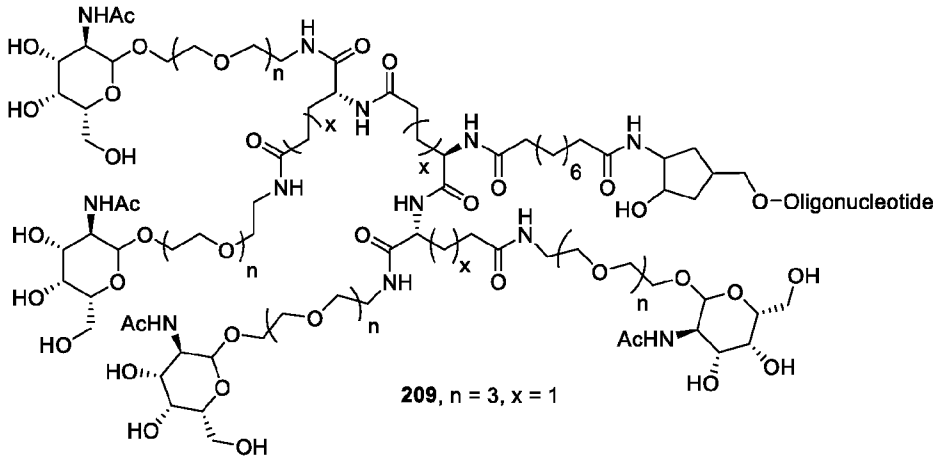
In one embodiment the compound is a compound or salt selected from the group consisting of:



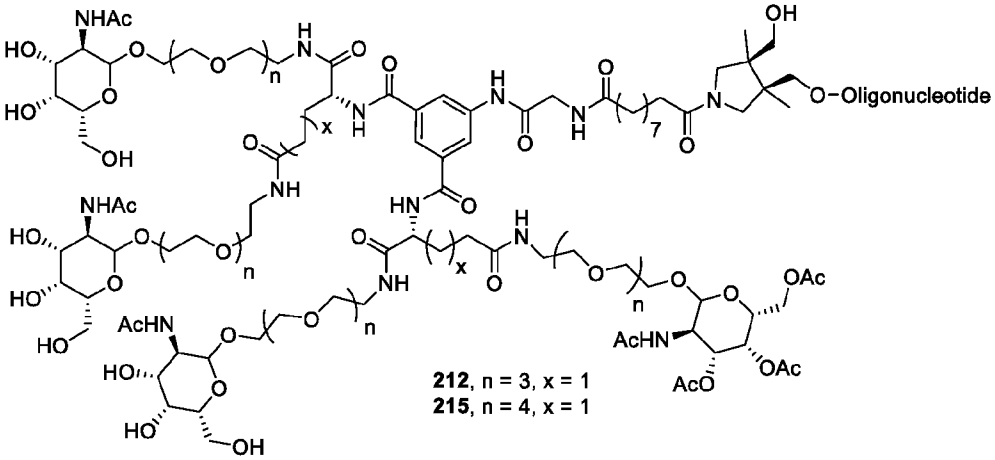





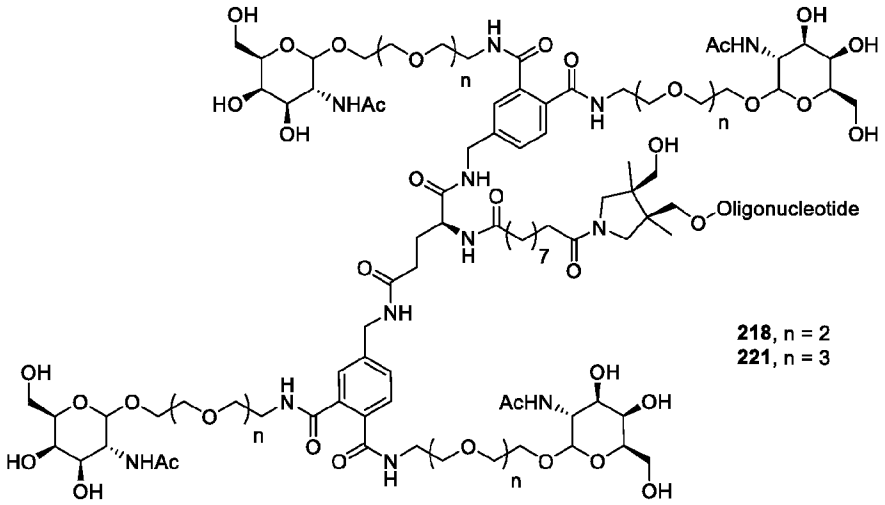

**203**, n = 3, x = 1
**206**, n = 4, x = 1

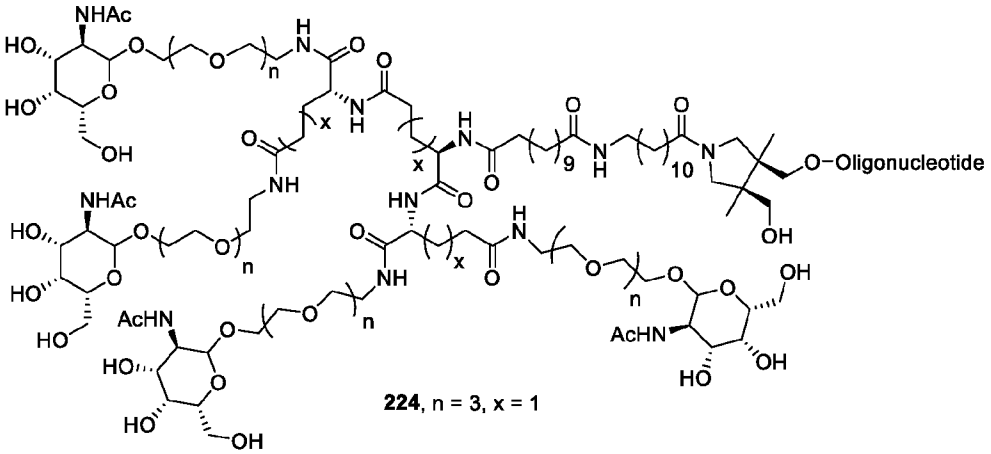


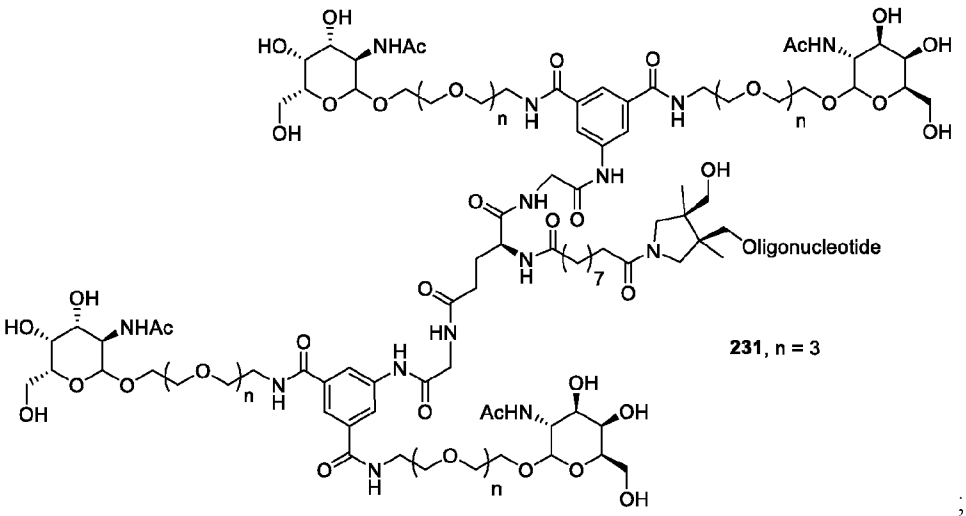

**209**, n = 3, x = 1




**212**, n = 3, x = 1
**215**, n = 4, x = 1



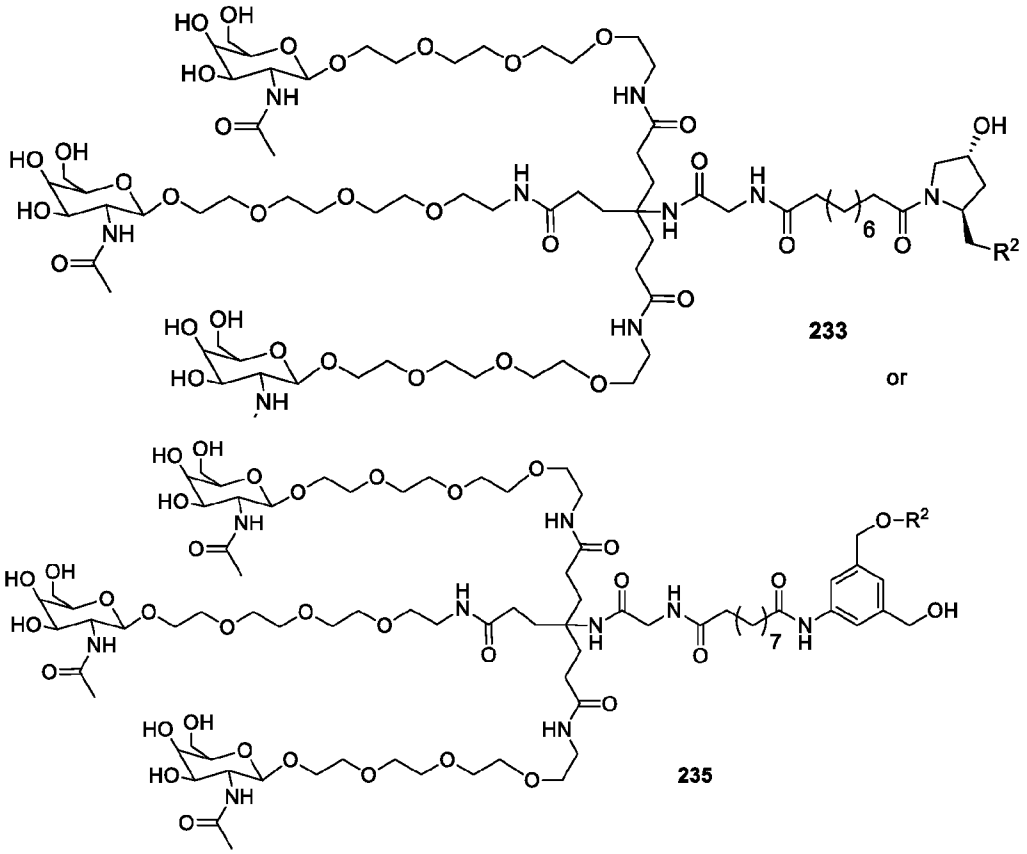




and ;

and pharmaceutically acceptable salts thereof.

In one embodiment the compound is:



233

or



235

or a salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein.

In one embodiment the compound is z conjugate of Formula X:

A-B-C

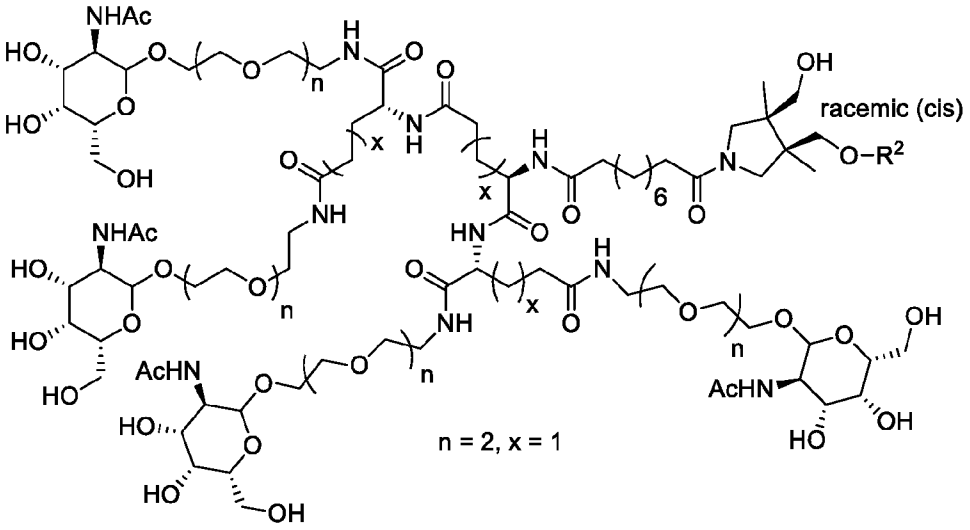
**(X)**

wherein A is a targeting ligand;

B is an optional linker; and

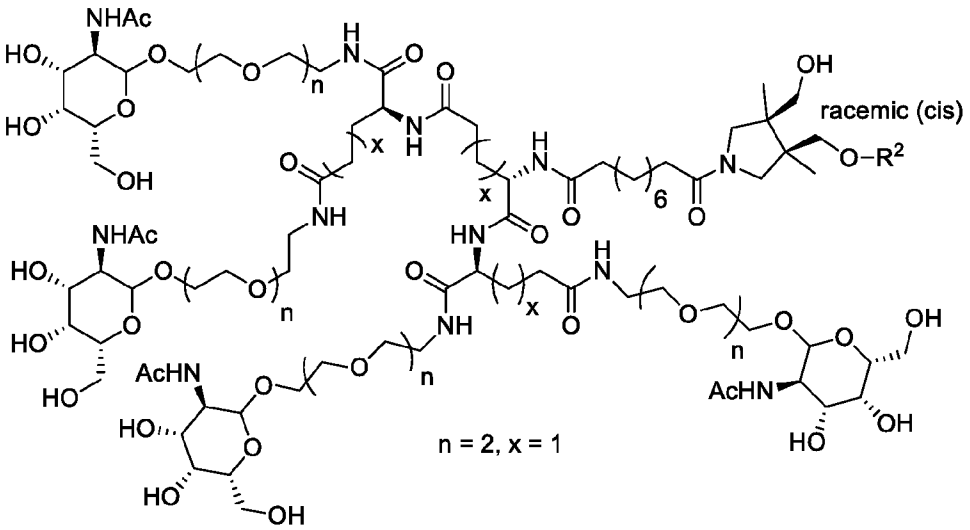
C is an siRNA molecule described herein.

In one embodiment, the compound is a compound of formula:



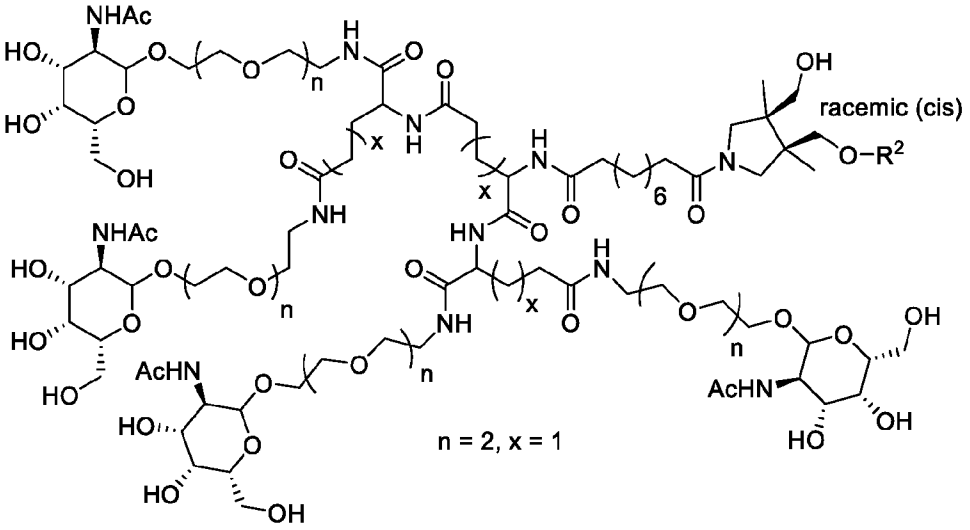

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



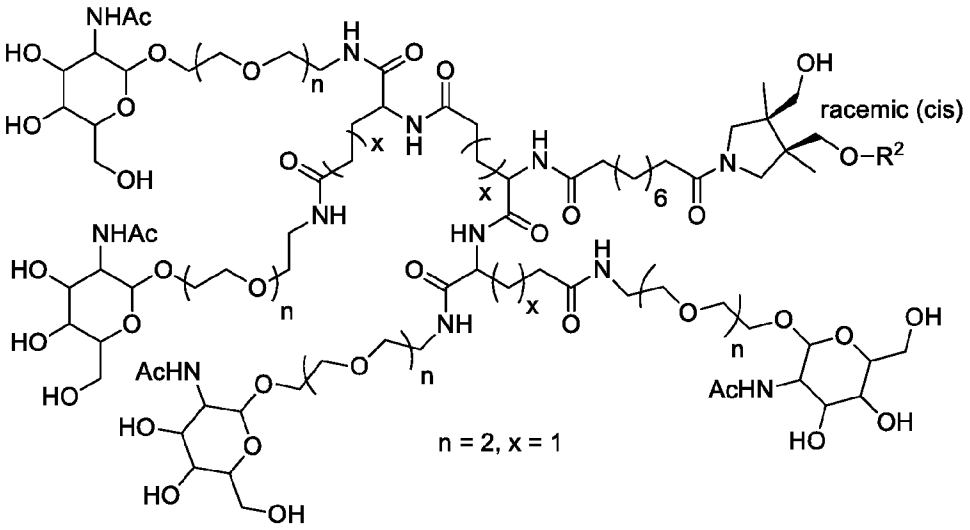

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment the compound is a compound of formula:



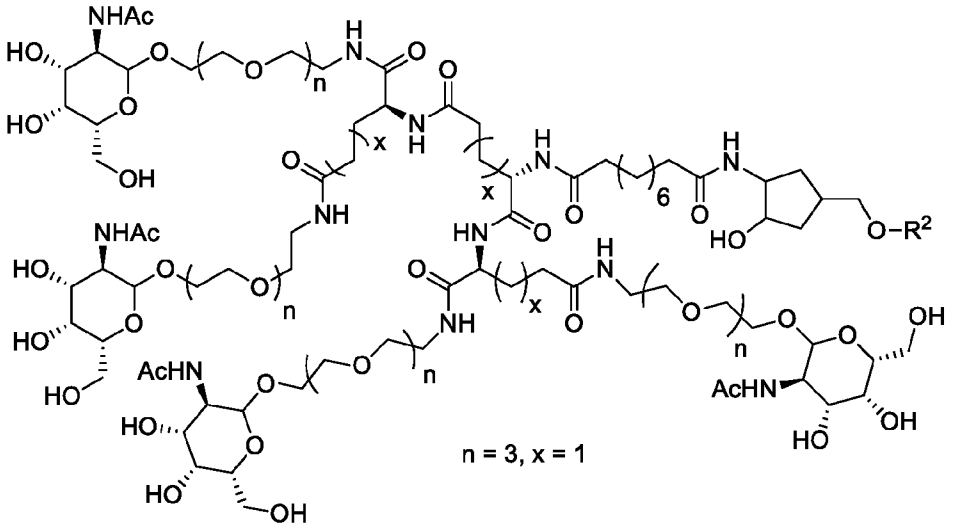

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



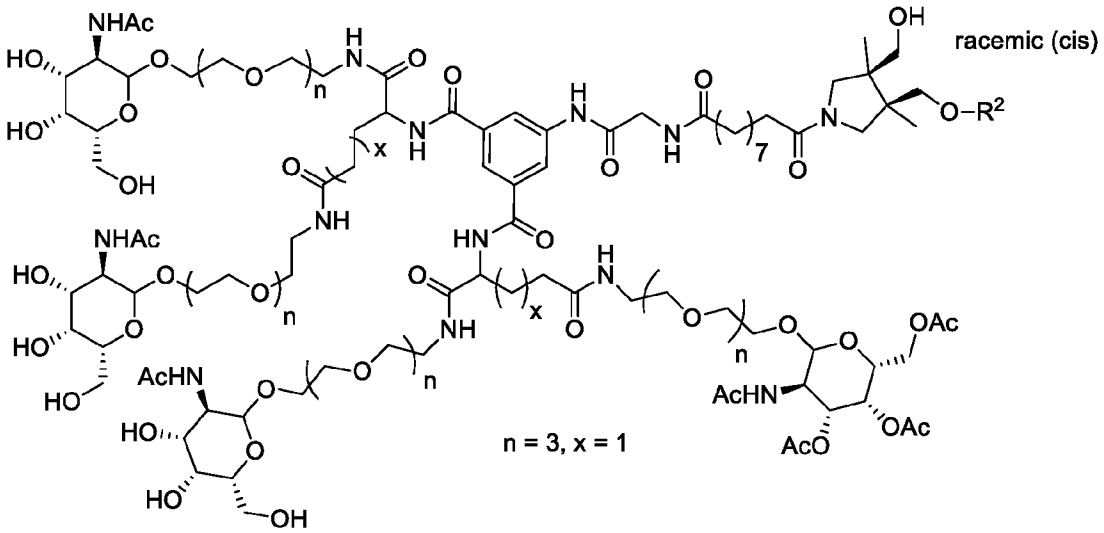

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



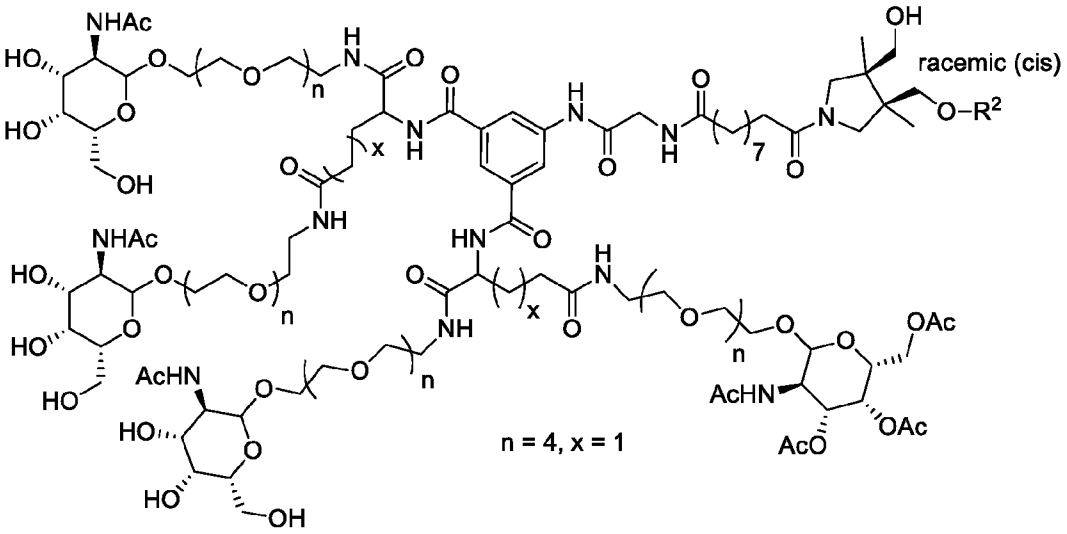

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



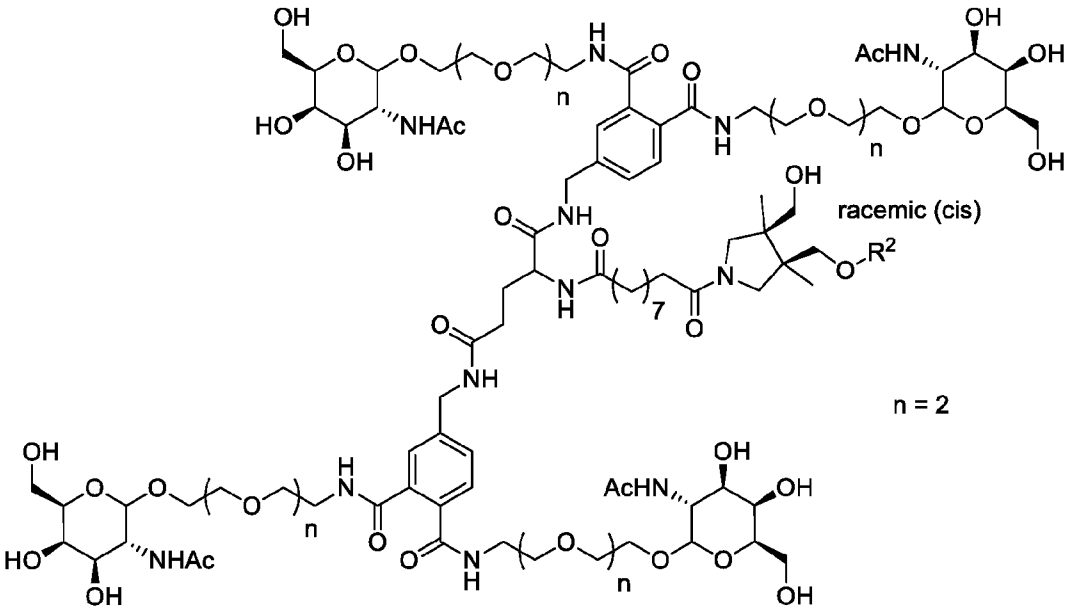

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



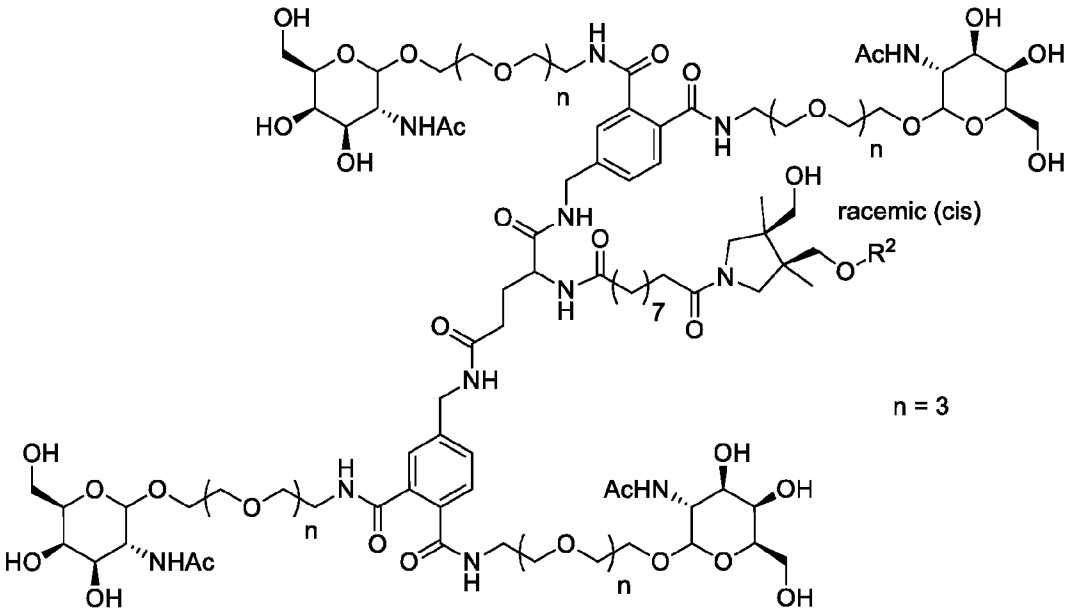

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



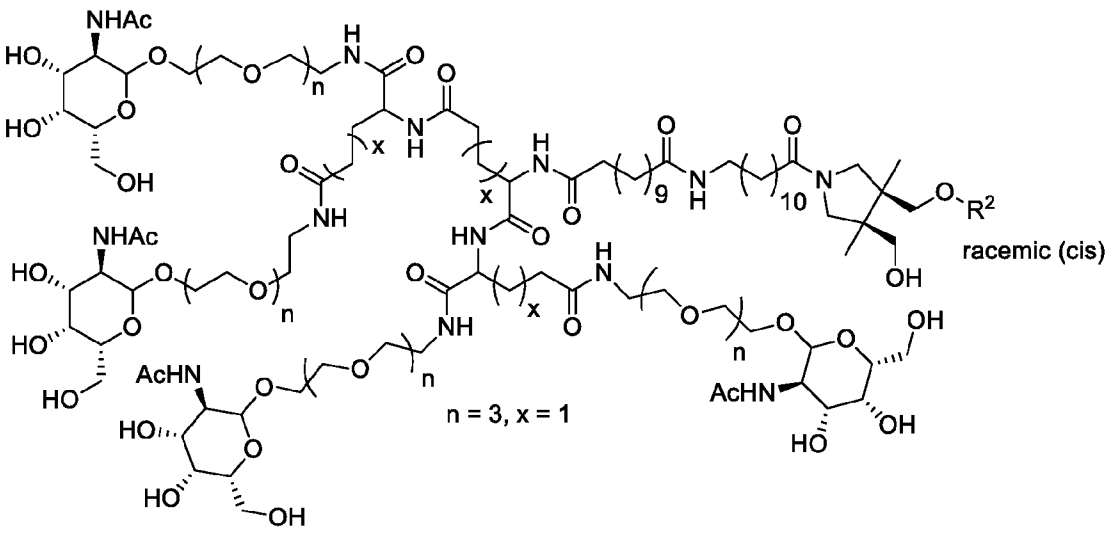

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment the compound is a compound of formula:



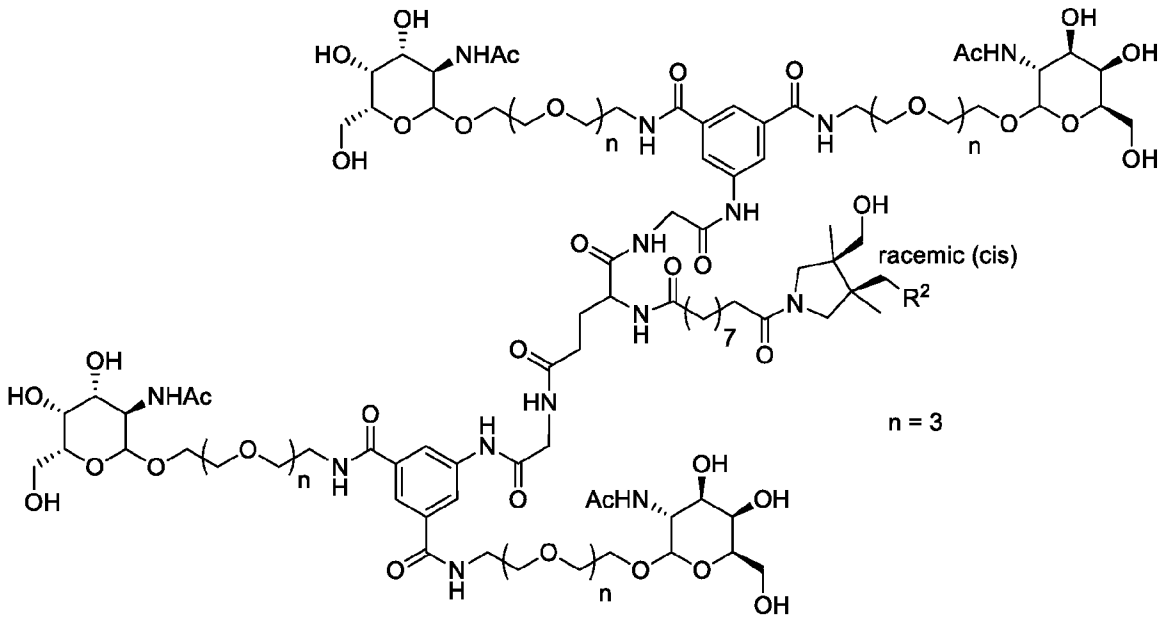

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



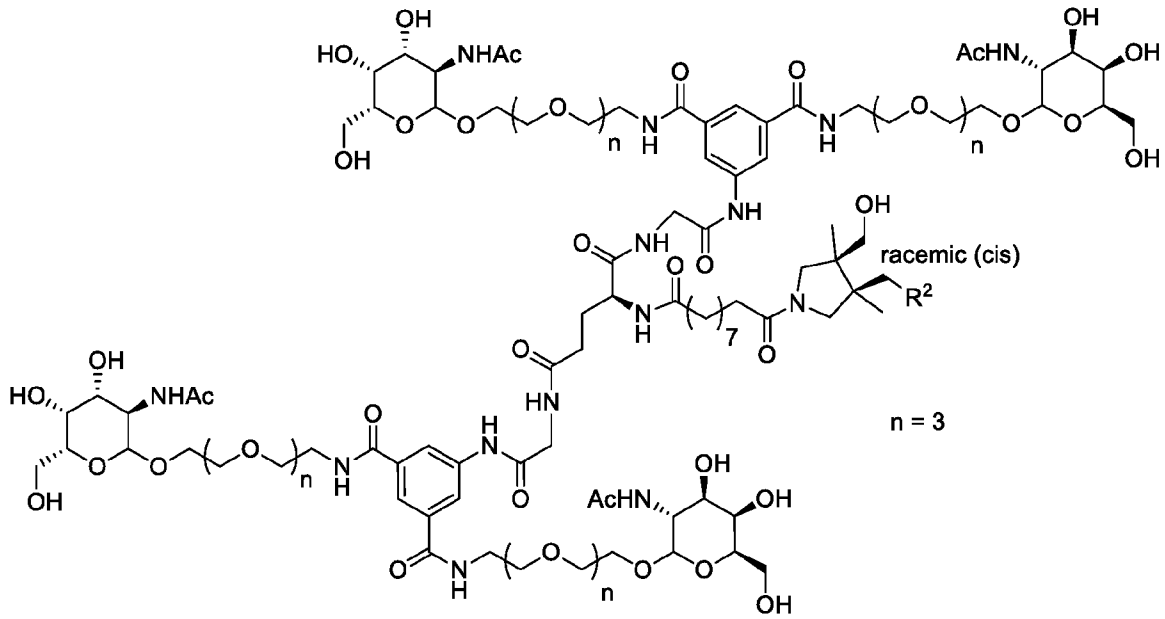

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment the compound is a compound of formula:



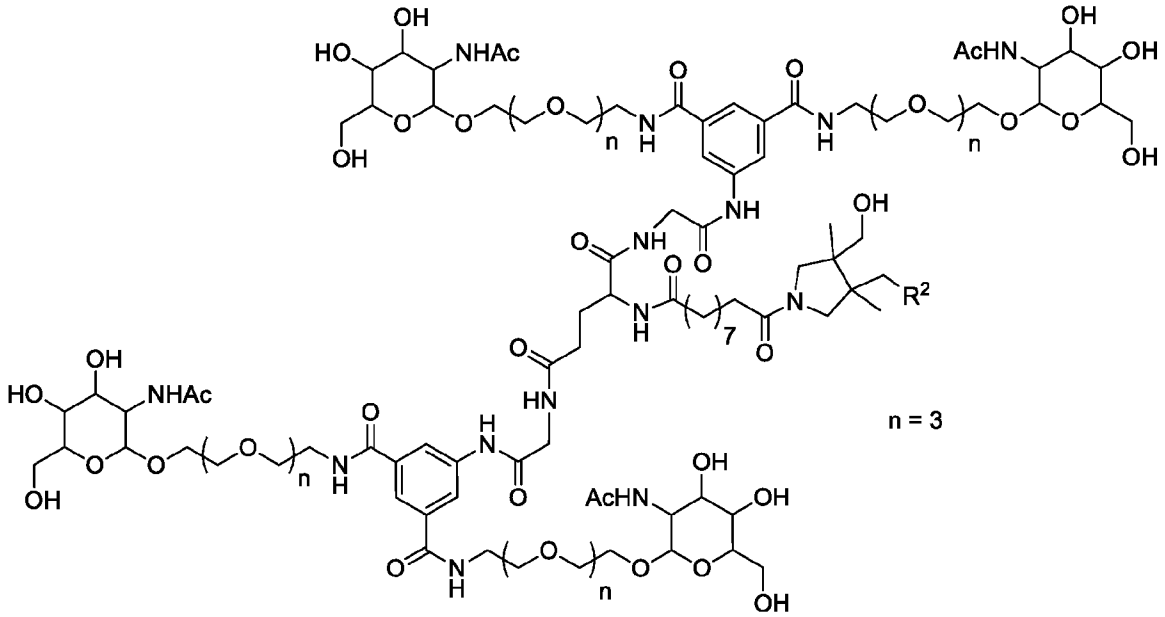

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



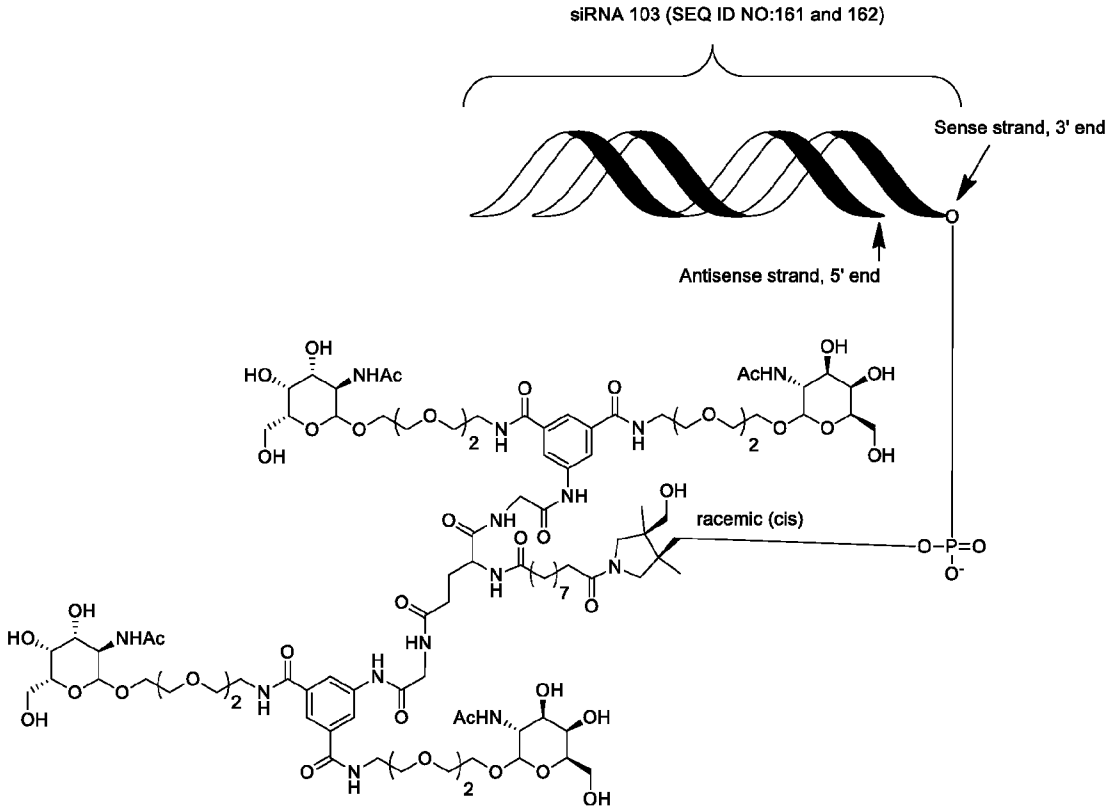

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



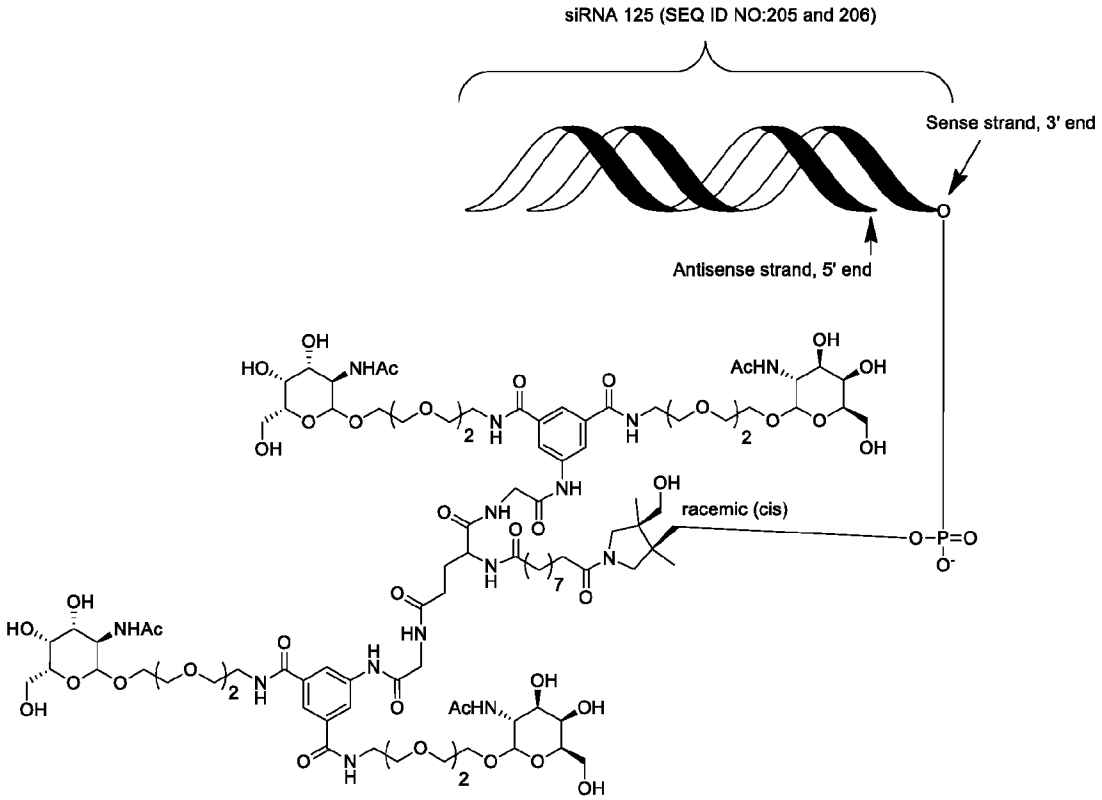

or a pharmaceutically acceptable salt thereof, wherein $R^2$ is a double stranded siRNA molecule selected from the double stranded siRNA molecules described herein (e.g., in Tables A or B).

In one embodiment, the compound is a compound of formula:



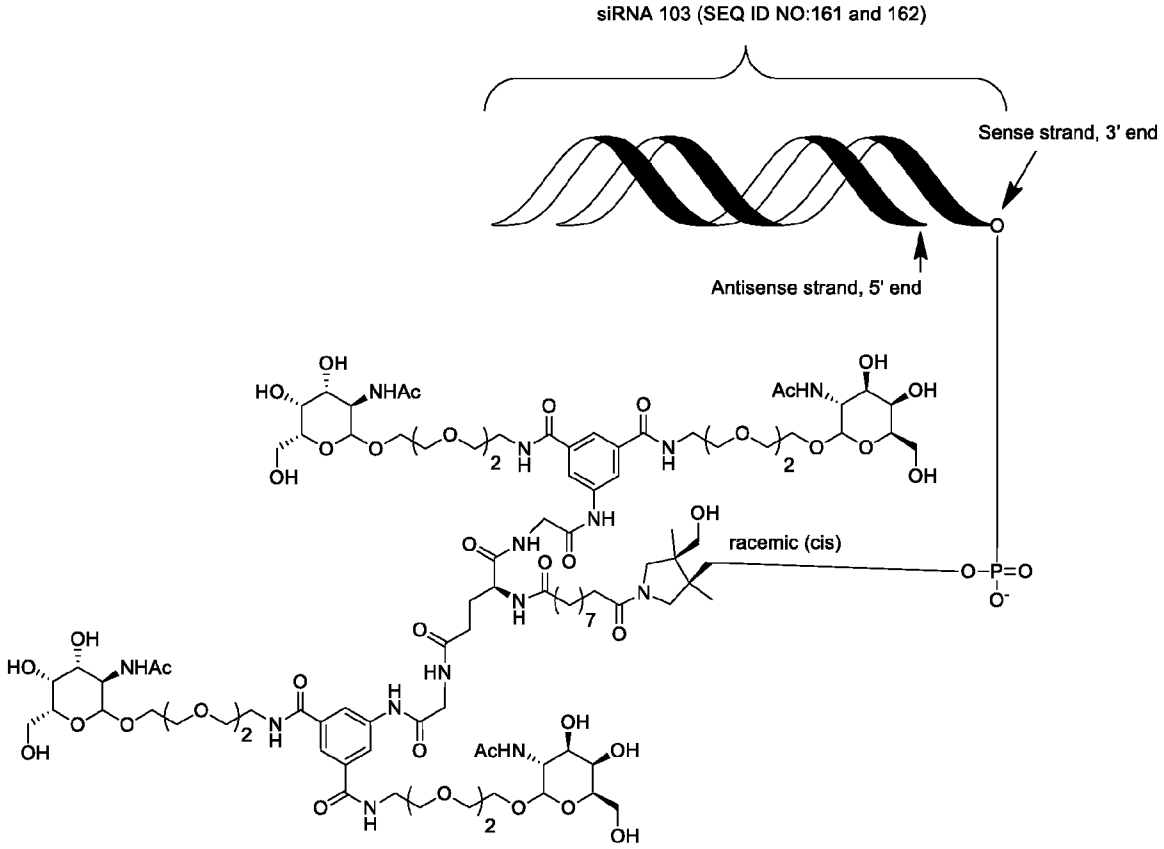

or a pharmaceutically acceptable salt thereof.

In one embodiment the compound is a compound of formula:



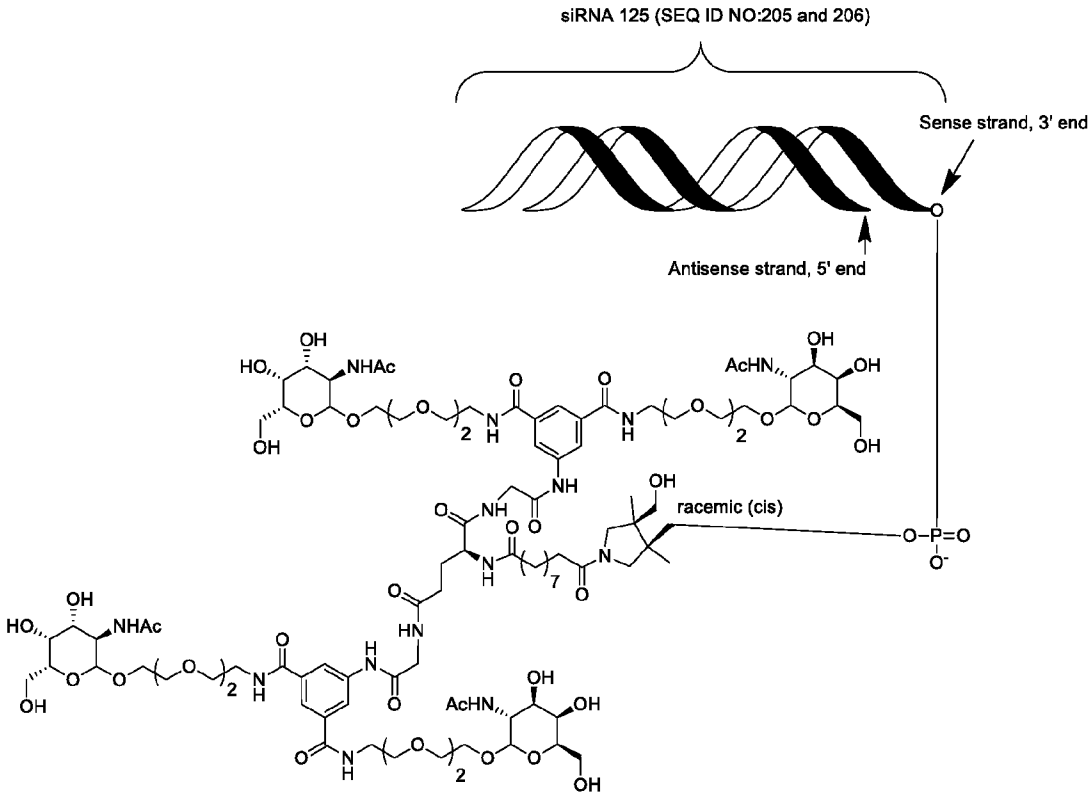

or a pharmaceutically acceptable salt thereof.

In one embodiment the compound is a compound of formula:



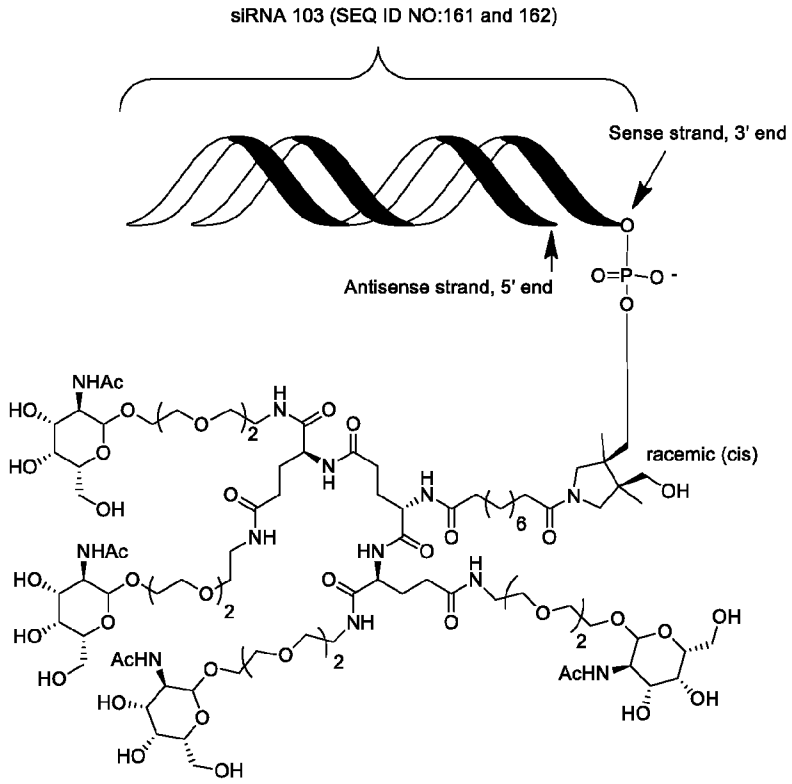

or a pharmaceutically acceptable salt thereof.

In one embodiment the compound is a compound of formula:



or a pharmaceutically acceptable salt thereof.

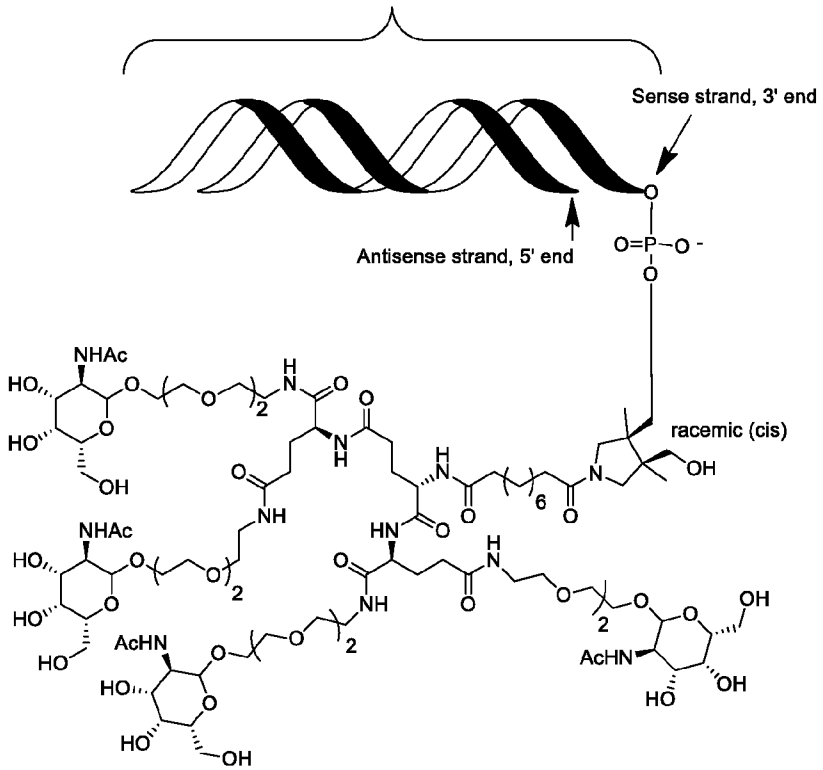
In one embodiment the compound is a compound of formula:




or a pharmaceutically acceptable salt thereof.

In one embodiment the compound is a compound of formula:

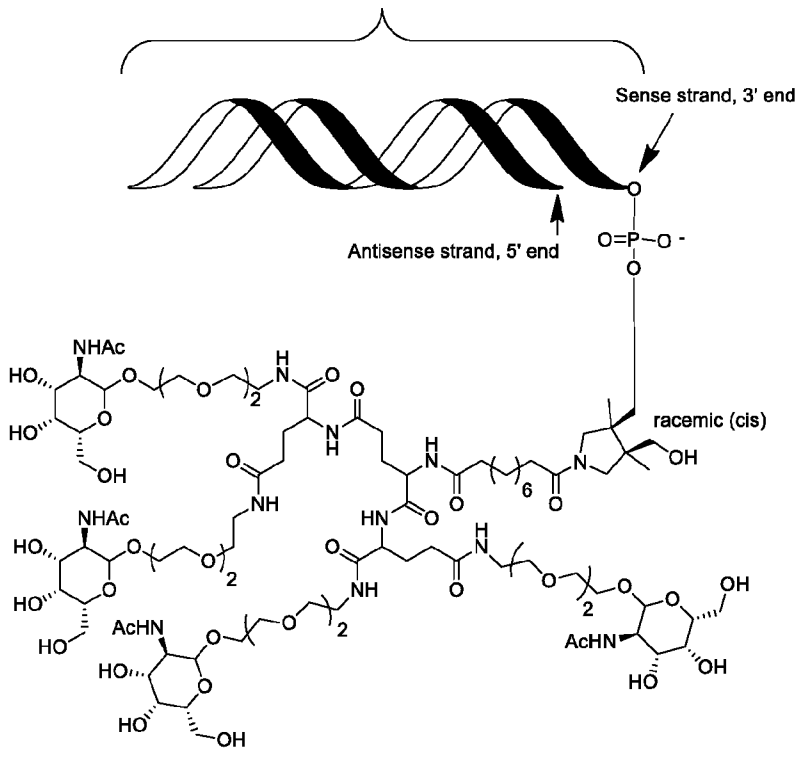
siRNA 125 (SEQ ID NO:205 and 206)




or a pharmaceutically acceptable salt thereof.

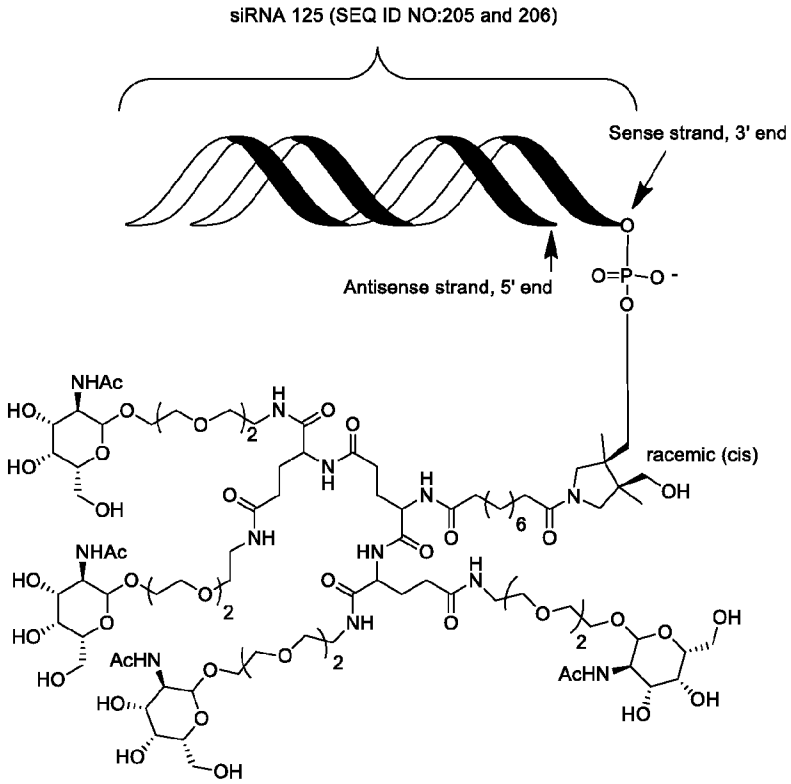
In one embodiment the compound is a compound of formula:

siRNA 103 (SEQ ID NO:161 and 162)



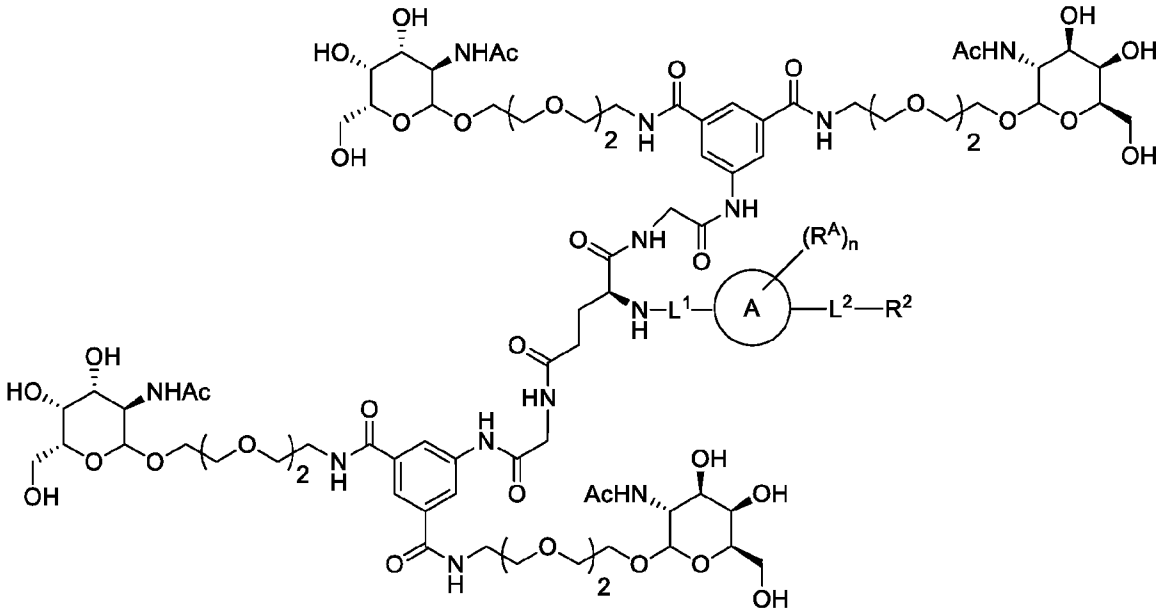
or a pharmaceutically acceptable salt thereof.

In one embodiment, the compound is a compound of formula:




or a pharmaceutically acceptable salt thereof.

In one embodiment the compound is a compound of formula (I):



(I)

wherein:

$L^1$ is absent or a linking group;

$L^2$ is absent or a linking group;

$R^2$ is a nucleic acid;

the ring A is absent, a 3-20 membered cycloalkyl, a 5-20 membered aryl, a 5-20 membered heteroaryl, or a 3-20 membered heterocycloalkyl;

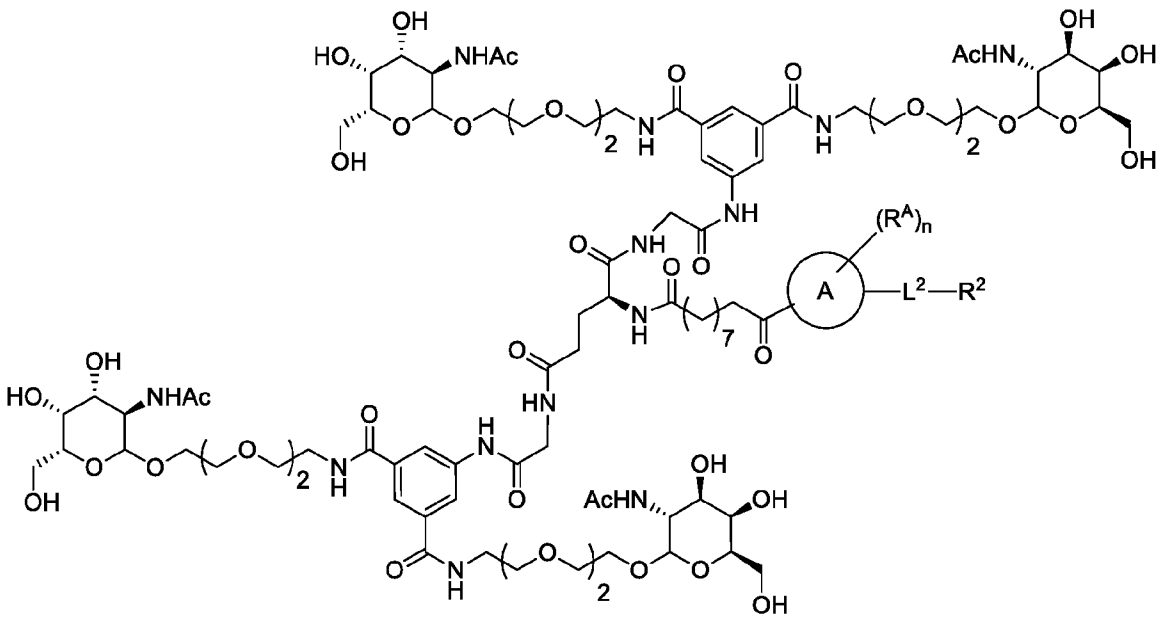
each $R^A$ is independently selected from the group consisting of hydrogen, hydroxy, CN, F, Cl, Br, I, -$C_{1-2}$ alkyl-$OR^B$, $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl; wherein the $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl are optionally substituted with one or more groups independently selected from halo, hydroxy, and $C_{1-3}$ alkoxy;

$R^B$ is hydrogen, a protecting group, a covalent bond to a solid support, or a bond to a linking group that is bound to a solid support; and

n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

or a salt thereof.

In one embodiment the compound is a compound of formula (Ih):



(Ih)

wherein:

$L^2$ is absent or a linking group;

$R^2$ is an oligonucleotide;

the ring A is absent, a 3-20 membered cycloalkyl, a 5-20 membered aryl, a 5-20 membered heteroaryl, or a 3-20 membered heterocycloalkyl;

each $R^A$ is independently selected from the group consisting of hydrogen, hydroxy, CN, F, Cl, Br, I, -$C_{1-2}$ alkyl-$OR^B$, $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl; wherein the $C_{1-10}$ alkyl

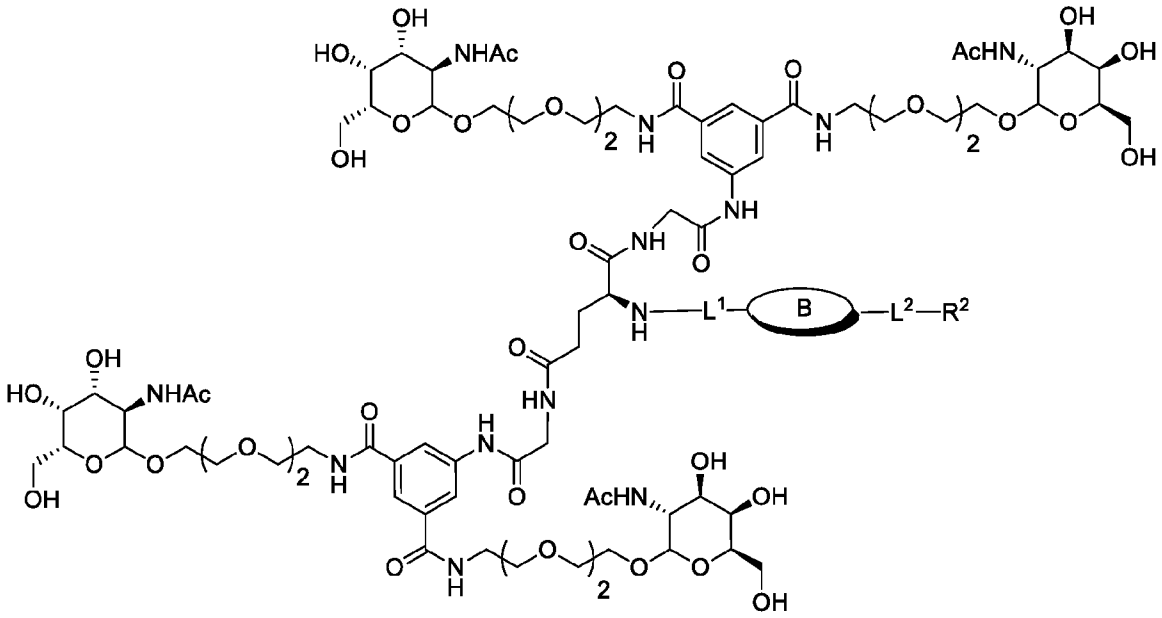
$C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl are optionally substituted with one or more groups independently selected from halo, hydroxy, and $C_{1-3}$ alkoxy;

$R^B$ is hydrogen, a protecting group, a covalent bond to a solid support, or a bond to a linking group that is bound to a solid support; and

n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

or a salt thereof.

In one embodiment the compound is a compound of formula (II):



(II)

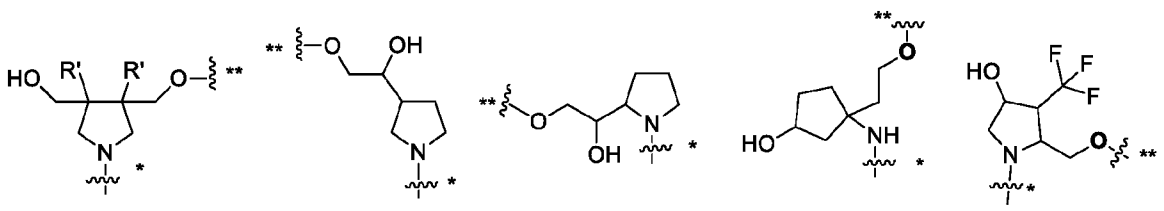
wherein:

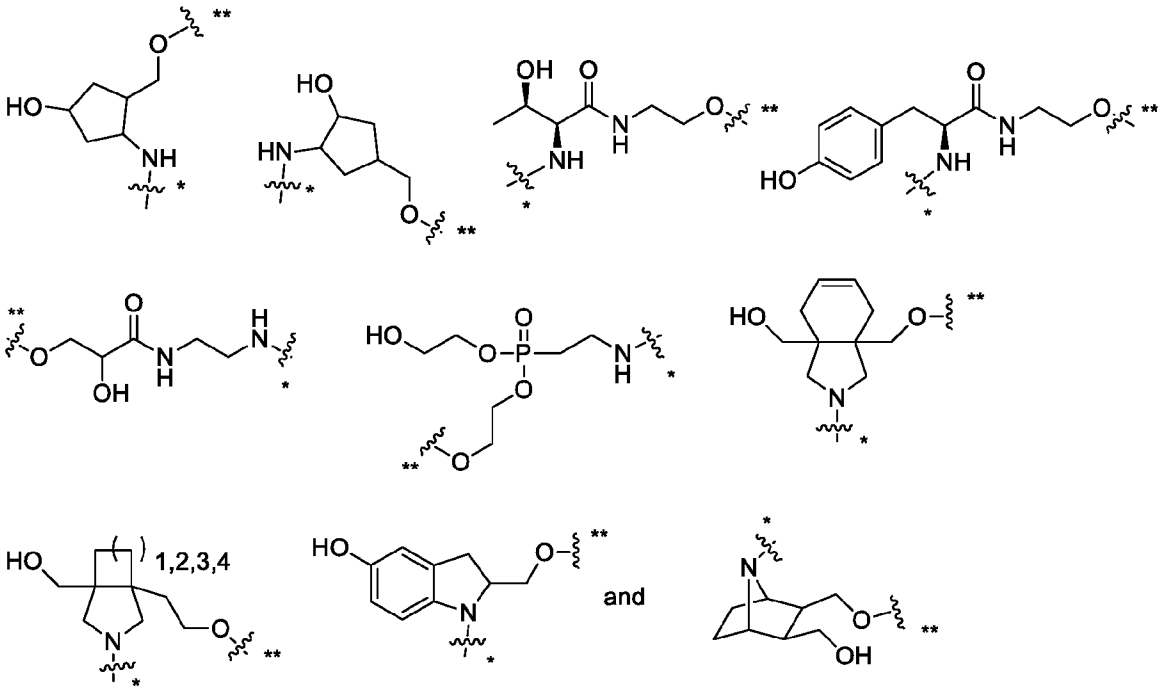
$L^1$ is absent or a linking group;

$L^2$ is absent or a linking group;

$R^2$ is an oligonucleotide;

B is divalent and is selected from the group consisting of:






wherein:

each R' is independently $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl; wherein the $C_{1-9}$ alkyl, $C_{2-9}$ alkenyl or $C_{2-9}$ alkynyl are optionally substituted with halo or hydroxyl;

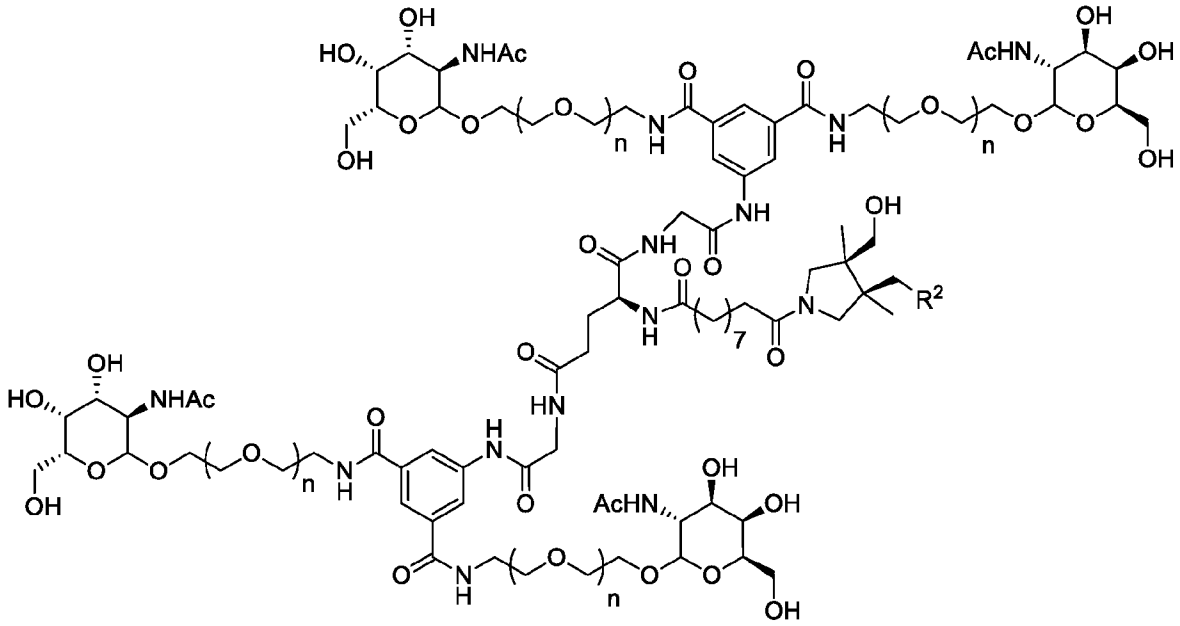
the valence marked with * is attached to $L^1$ or is attached to $R^1$ if $L^1$ is absent; and

the valence marked with ** is attached to $L^2$ or is attached to $R^2$ if $L^2$ is absent;

or a salt thereof.

In one embodiment $L^1$ and $L^2$ are independently a divalent, branched or unbranched, saturated or unsaturated, hydrocarbon chain, having from 1 to 50 carbon atoms, wherein one or more (e.g. 1, 2, 3, or 4) of the carbon atoms in the hydrocarbon chain is optionally replaced by –O-, -$NR^X$-, -$NR^X$-C(=O)-, -C(=O)-$NR^X$- or –S-, and wherein $R^X$ is hydrogen or (C1-C6)alkyl, and wherein the hydrocarbon chain, is optionally substituted with one or more substituents selected from (C1-C6)alkoxy, (C3-C6)cycloalkyl, (C1-C6)alkanoyl, (C1-C6)alkanoyloxy, (C1-C6)alkoxycarbonyl, (C1-C6)alkylthio, azido, cyano, nitro, halo, hydroxy, oxo (=O), carboxy, aryl, aryloxy, heteroaryl, and heteroaryloxy.

In one embodiment the compound is:

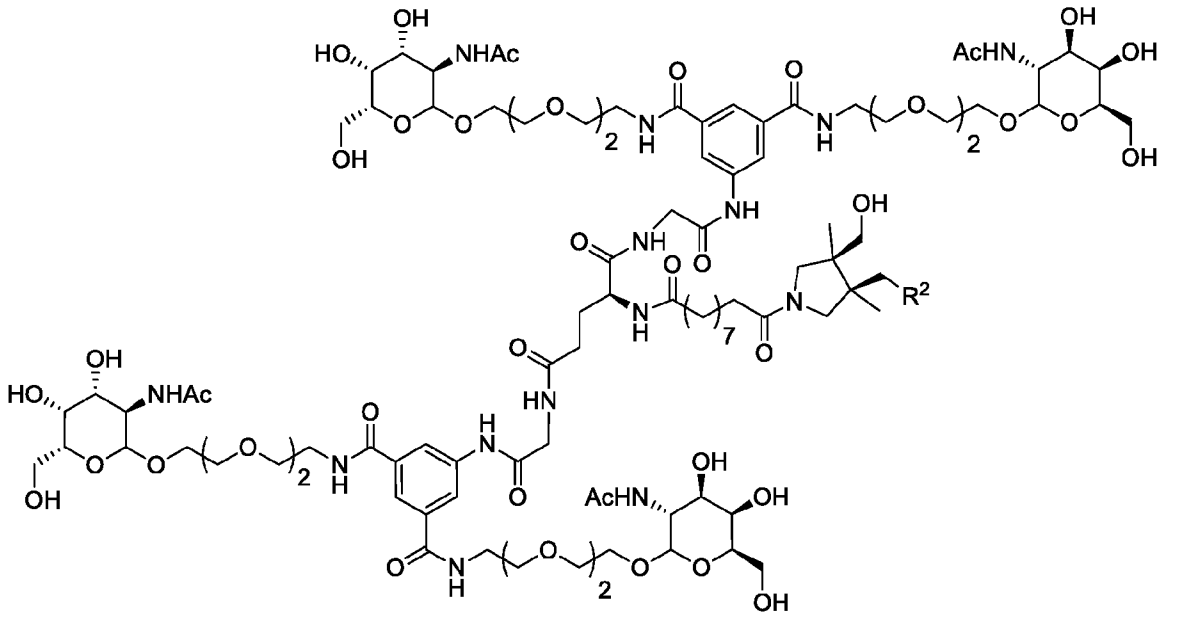


or a salt thereof wherein $R^2$ is an oligonucleotide.

In one embodiment, n is 2, 3, 4, 5 or 6.

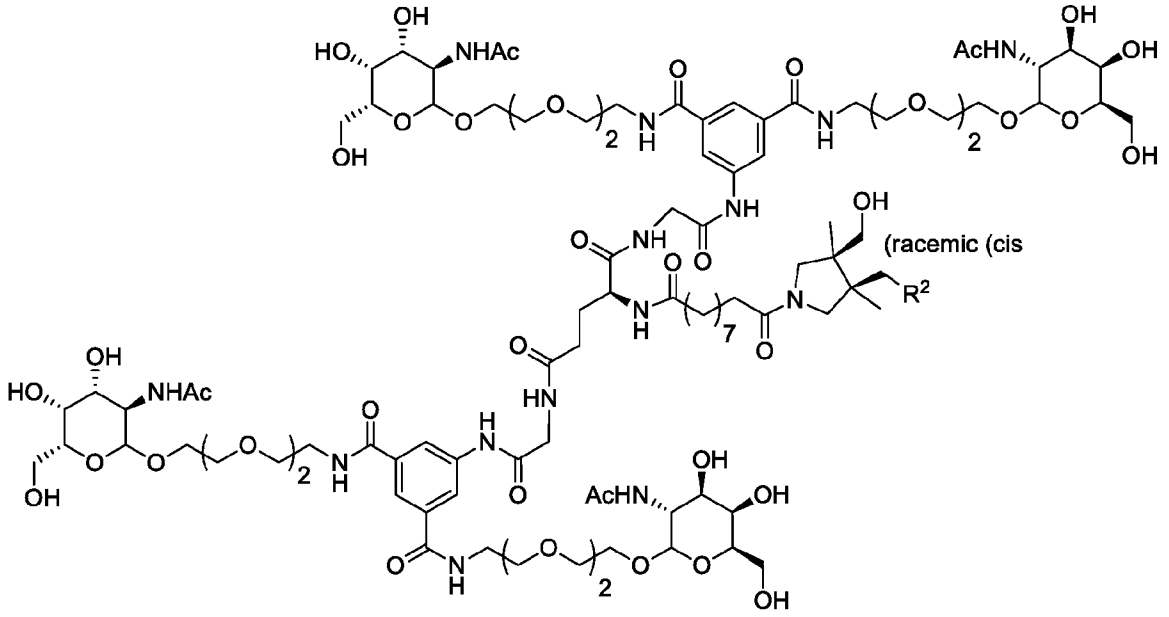
In one embodiment $R^2$ is attached through the oxygen of a phosphate at the 3'-end of the sense strand.

In one embodiment the compound is:



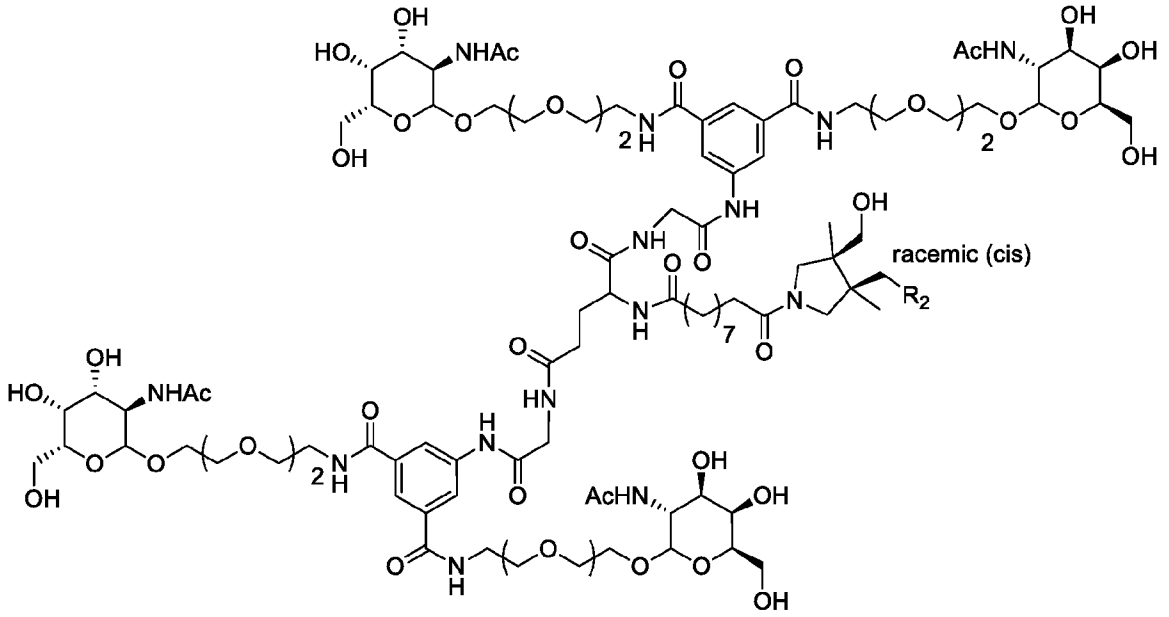
or a salt thereof wherein $R^2$ is a double stranded siRNA molecule as described in Table B that is attached through the oxygen of a phosphate at the 3'-end of the sense strand.

In one embodiment the compound is:



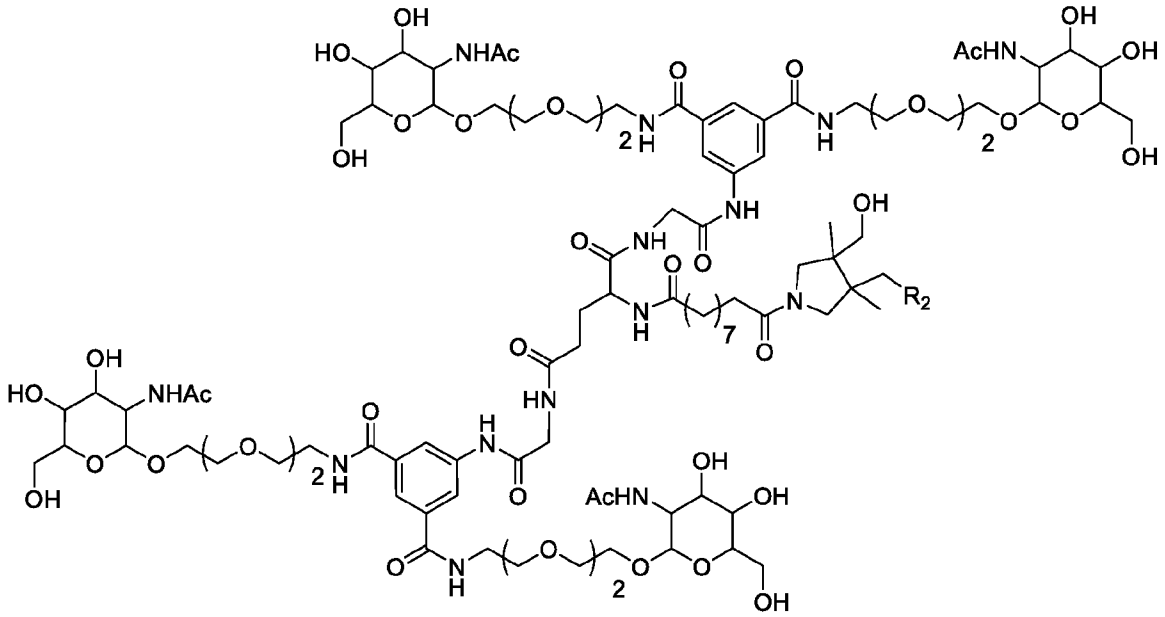

or a salt thereof wherein $R^2$ is a nucleic acid.

In one embodiment the compound is:




or a salt thereof wherein $R^2$ is a nucleic acid.

In one embodiment the compound is:



or a salt thereof wherein $R^2$ is a nucleic acid.

*Certain Definitions for Conjugate Embodiments*

The term "synthetic activating group" refers to a group that can be attached to an atom to activate that atom to allow it to form a covalent bond with another reactive group. It is understood that the nature of the synthetic activating group may depend on the atom that it is activating. For example, when the synthetic activating group is attached to an oxygen atom, the synthetic activating group is a group that will activate that oxygen atom to form a bond (e.g. an ester, carbamate, or ether bond) with another reactive group. Such synthetic activating groups are known. Examples of synthetic activating groups that can be attached to an oxygen atom include, but are not limited to, acetate, succinate, triflate, and mesylate. When the synthetic activating group is attached to an oxygen atom of a carboxylic acid, the synthetic activating group can be a group that is derivable from a known coupling reagent (e.g. a known amide coupling reagent). Such coupling reagents are known. Examples of such coupling reagents include, but are not limited to, N,N'-Dicyclohexylcarbodimide (DCC), hydroxybenzotriazole (HOBt), N-(3-Dimethylaminopropyl)-N'-ethylcarbonate (EDC), (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) or O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU).

As used herein, the term "alkyl", by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain hydrocarbon radical, having the number of carbon

atoms designated (i.e., $C_{1-8}$ means one to eight carbons). Examples of alkyl groups include methyl, ethyl, n-propyl, iso-propyl, n-butyl, t-butyl, iso-butyl, sec-butyl, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. The term "alkenyl" refers to an unsaturated alkyl radical having one or more double bonds. Similarly, the term "alkynyl" refers to an unsaturated alkyl radical having one or more triple bonds. Examples of such unsaturated alkyl groups include vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers.

The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane (including straight and branched alkanes), as exemplified by $-CH_2CH_2CH_2CH_2-$ and $-CH(CH_3)CH_2CH_2-$.

The term "cycloalkyl," "carbocyclic," or "carbocycle" refers to hydrocarbon ringsystem having 3 to 20 overall number of ring atoms (e.g., 3-20 membered cycloalkyl is a cycloalkyl with 3 to 20 ring atoms, or $C_{3-20}$ cycloalkyl is a cycloalkyl with 3-20 carbon ring atoms) and for a 3-5 membered cycloalkyl being fully saturated or having no more than one double bond between ring vertices and for a 6 membered cycloalkyl or larger being fully saturated or having no more than two double bonds between ring vertices. As used herein, "cycloalkyl," "carbocyclic," or "carbocycle" is also meant to refer to bicyclic, polycyclic and spirocyclic hydrocarbon ring system, such as, for example, bicyclo[2.2.1]heptane, pinane, bicyclo[2.2.2]octane, adamantane, norborene, spirocyclic $C_{5-12}$ alkane, etc. As used herein, the terms, "alkenyl," "alkynyl," "cycloalkyl,", "carbocycle," and "carbocyclic," are meant to include mono and polyhalogenated variants thereof.

The term "heterocycloalkyl," "heterocyclic," or "heterocycle" refers to a saturated or partially unsaturated ring system radical having the overall having from 3-20 ring atoms (e.g., 3-20 membered heterocycloalkyl is a heterocycloalkyl radical with 3-20 ring atoms, a $C_{2-19}$ heterocycloalkyl is a heterocycloalkyl having 3-10 ring atoms with between 2-19 ring atoms being carbon) that contain from one to ten heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, nitrogen atom(s) are optionally quaternized, as ring atoms. Unless otherwise stated, a "heterocycloalkyl," "heterocyclic," or "heterocycle" ring can be a monocyclic, a bicyclic, spirocyclic or a polycylic ring system. Non limiting examples of "heterocycloalkyl," "heterocyclic," or "heterocycle" rings include pyrrolidine, piperidine, N-methylpiperidine, imidazolidine, pyrazolidine, butyrolactam, valerolactam, imidazolidinone, hydantoin, dioxolane, phthalimide, piperidine, pyrimidine-2,4(1H,3H)-dione, 1,4-dioxane, morpholine, thiomorpholine, thiomorpholine-S-oxide, thiomorpholine-S,S-oxide, piperazine, pyran, pyridone, 3-pyrroline, thiopyran, pyrone, tetrahydrofuran, tetrhydrothiophene,

quinuclidine, tropane, 2-azaspiro[3.3]heptane, (1R,5S)-3-azabicyclo[3.2.1]octane, (1s,4s)-2-azabicyclo[2.2.2]octane, (1R,4R)-2-oxa-5-azabicyclo[2.2.2]octane and the like A "heterocycloalkyl," "heterocyclic," or "heterocycle" group can be attached to the remainder of the molecule through one or more ring carbons or heteroatoms. A "heterocycloalkyl," "heterocyclic," or "heterocycle" can include mono- and poly-halogenated variants thereof.

The terms "alkoxy," and "alkylthio", are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom ("oxy") or thio group, and further include mono- and poly-halogenated variants thereof.

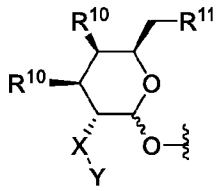
The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. The term "(halo)alkyl" is meant to include both a "alkyl" and "haloalkyl" substituent. Additionally, the term "haloalkyl," is meant to include monohaloalkyl and polyhaloalkyl. For example, the term "$C_{1-4}$ haloalkyl" is mean to include trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, difluoromethyl, and the like.

The term "aryl" means a carbocyclic aromatic group having 6-14 carbon atoms, whether or not fused to one or more groups. Examples of aryl groups include phenyl, naphthyl, biphenyl and the like unless otherwise stated.

The term "heteroaryl" refers to aryl ring(s) that contain from one to five heteroatoms selected from N, O, and S, wherein the nitrogen and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Examples of heteroaryl groups include pyridyl, pyridazinyl, pyrazinyl, pyrimindinyl, triazinyl, quinolinyl, quinoxalinyl, quinazolinyl, cinnolinyl, phthalaziniyl, benzotriazinyl, purinyl, benzimidazolyl, benzopyrazolyl, benzotriazolyl, benzisoxazolyl, isobenzofuryl, isoindolyl, indolizinyl, benzotriazinyl, thienopyridinyl, thienopyrimidinyl, pyrazolopyrimidinyl, imidazopyridines, benzothiaxolyl, benzofuranyl, benzothienyl, indolyl, quinolyl, isoquinolyl, isothiazolyl, pyrazolyl, indazolyl, pteridinyl, imidazolyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, thiadiazolyl, pyrrolyl, thiazolyl, furyl, thienyl and the like.

The term saccharide includes monosaccharides, disaccharides and trisaccharides. The term includes glucose, sucrose fructose, galactose and ribose, as well as deoxy sugars such as deoxyribose and amino sugar such as galactosamine. Saccharide derivatives can conveniently be prepared as described in International Patent Applications Publication Numbers WO 96/34005 and 97/03995. A saccharide can conveniently be linked to the remainder of a compound of formula I through an ether bond, a thioether bond (e.g. an S-glycoside), an amine

nitrogen (e.g., an *N*-glycoside ), or a carbon-carbon bond (e.g. a C-glycoside). In one embodiment the saccharide can conveniently be linked to the remainder of a compound of formula I through an ether bond. In one embodiment the term saccharide includes a group of the formula:



wherein:

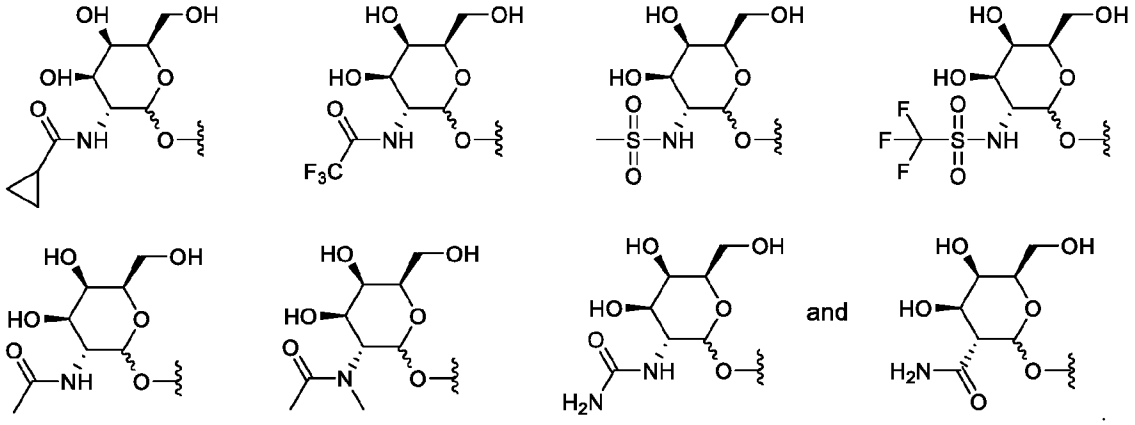
X is $NR^3$, and Y is selected from $-(C=O)R^4$, $-SO_2R^5$, and $-(C=O)NR^6R^7$; or X is -(C=O)- and Y is $NR^8R^9$;

$R^3$ is hydrogen or $(C_1\text{-}C_4)$alkyl;

$R^4$, $R^5$, $R^6$, $R^7$, $R^8$ and $R^9$ are each independently selected from the group consisting of hydrogen, $(C_1\text{-}C_8)$alkyl, $(C_1\text{-}C_8)$haloalkyl, $(C_1\text{-}C_8)$alkoxy and $(C_3\text{-}C_6)$cycloalkyl that is optionally substituted with one or more groups independently selected from the group consisting of halo, $(C_1\text{-}C_4)$alkyl, $(C_1\text{-}C_4)$haloalkyl, $(C_1\text{-}C_4)$alkoxy and $(C_1\text{-}C_4)$haloalkoxy;

$R^{10}$ is -OH, $-NR^8R^9$ or – F; and

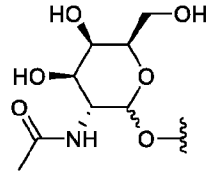
$R^{11}$ is -OH, $-NR^8R^9$, -F or 5 membered heterocycle that is optionally substituted with one or more groups independently selected from the group consisting of halo, hydroxyl, carboxyl, amino, $(C_1\text{-}C_4)$alkyl, $(C_1\text{-}C_4)$haloalkyl, $(C_1\text{-}C_4)$alkoxy and $(C_1\text{-}C_4)$haloalkoxy. In another embodiment the saccharide can be selected from the group consisting of:



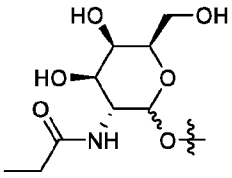
and



In another embodiment the saccharide can be:




or




*N*-Acetylgalactosamine (GalNAc) GalPro.

**Formulation and Administration of Conjugates and/or Additional Therapeutic Agents**

The agents (*i.e.*, conjugates and additional therapeutic agents) can be formulated for and administered using any acceptable route of administration depending on the agent selected. For example, suitable routes include, but are not limited to, oral, sublingual, buccal, topical, transdermal, parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration.

When a combination of agents is administered, it will be understood that the agents can be formulated together in a single preparation or that they can be formulated separately and, thus, administered separately, either simultaneously or sequentially. In one embodiment, when the agents are administered sequentially (e.g. at different times), the agents may be administered so that their biological effects overlap (i.e. each agent is producing a biological effect at a single given time).

The agents can be individually formulated by mixing at ambient temperature at the appropriate pH, and at the desired degree of purity, with physiologically acceptable carriers, *i.e.*, carriers that are non-toxic to recipients at the dosages and concentrations employed. The pH of the formulation depends mainly on the particular use and the concentration of compound, but may typically range anywhere from about 3 to about 8. The agents ordinarily will be stored as a solid composition, although lyophilized formulations or aqueous solutions are acceptable.

Compositions comprising the agents can be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular human being treated, the clinical condition of the individual patient, the cause of the disorder, the site of administration, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

The agents may be administered in any convenient administrative form, *e.g.*, tablets, powders, capsules, solutions, dispersions, suspensions, syrups, sprays, suppositories, gels, emulsions, patches, etc. Such compositions may contain components conventional in pharmaceutical preparations, *e.g.*, diluents, carriers, pH modifiers, sweeteners, bulking agents,

and further active agents. If parenteral administration is desired, the compositions will be sterile and in a solution or suspension form suitable for injection or infusion.

Suitable carriers and excipients are well known to those skilled in the art and are described in detail in, *e.g.*, Ansel, Howard C., et al., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems. Philadelphia: Lippincott, Williams & Wilkins, 2004; Gennaro, Alfonso R., et al. Remington: The Science and Practice of Pharmacy. Philadelphia: Lippincott, Williams & Wilkins, 2000; and Rowe, Raymond C. Handbook of Pharmaceutical Excipients. Chicago, Pharmaceutical Press, 2005. The formulations may also include one or more buffers, stabilizing agents, surfactants, wetting agents, lubricating agents, emulsifiers, suspending agents, preservatives, antioxidants, opaquing agents, glidants, processing aids, colorants, sweeteners, perfuming agents, flavoring agents, diluents and other known additives to provide an elegant presentation of the drug or aid in the manufacturing of the pharmaceutical product (*i.e.*, medicament).

The agents are typically dosed at least at a level to reach the desired biological effect. Thus, an effective dosing regimen will dose at least a minimum amount that reaches the desired biological effect, or biologically effective dose, however, the dose should not be so high as to outweigh the benefit of the biological effect with unacceptable side effects. Therefore, an effective dosing regimen will dose no more than the maximum tolerated dose ("MTD"). The maximum tolerated dose is defined as the highest dose that produces an acceptable incidence of dose-limiting toxicities ("DLT"). Doses that cause an unacceptable rate of DLT are considered non-tolerated. Typically, the MTD for a particular schedule is established in phase 1 clinical trials. These are usually conducted in patients by starting at a safe starting dose of 1/10 the severe toxic dose ("STD10") in rodents (on a $mg/m^2$ basis) and accruing patients in cohorts of three, escalating the dose according to a modified Fibonacci sequence in which ever higher escalation steps have ever decreasing relative increments (e.g., dose increases of 100%, 65%, 50%, 40%, and 30% to 35% thereafter). The dose escalation is continued in cohorts of three patients until a non-tolerated dose is reached. The next lower dose level that produces an acceptable rate of DLT is considered to be the MTD.

The amount of the agents administered will depend upon the particular agent used, the strain of HBV being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.2 to 2.0 grams per day.

**Kits**

The present invention also provides kits comprising:

a) an HBV antigen inhibitor;

b) instructions for administering the inhibitor to a hepatitis B virus (HBV) infected patient determined to have a C/C genotype at rs12079860.

In certain embodiments the HBV antigen inhibitor is an oligonucleotide (e.g., an siRNA). In certain embodiments, the oligonucleotide is comprised within a lipid nanoparticle formulation. Thus, the present invention also provides lipid particles in kit form. In some embodiments, the kit comprises a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents, such as siRNA molecules and the individual lipid components of the particles). Preferably, the kit comprises a container (*e.g.*, a vial or ampoule) which holds the lipid particles of the invention, wherein the particles are produced by one of the processes set forth herein. In certain embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the particle compositions of the invention, either as a suspension in a pharmaceutically acceptable carrier or in dehydrated form, with instructions for their rehydration (if lyophilized) and administration.

The formulations of the present invention can be tailored to preferentially target particular cells, tissues, or organs of interest. Preferential targeting of a nucleic acid-lipid particle may be carried out by controlling the composition of the lipid particle itself. In particular embodiments, the kits of the invention comprise these lipid particles, wherein the particles are present in a container as a suspension or in dehydrated form.

In certain instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

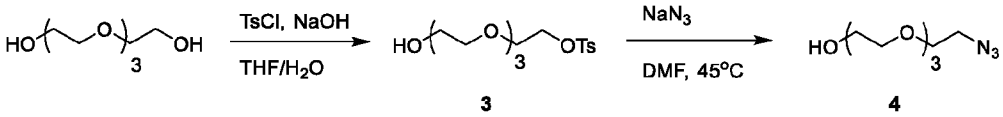
In certain embodiments, the HBV antigen inhibitor is conjugated to a targeting moiety. Thus, the present invention also provides conjugates described herein in kit form.

**Examples**

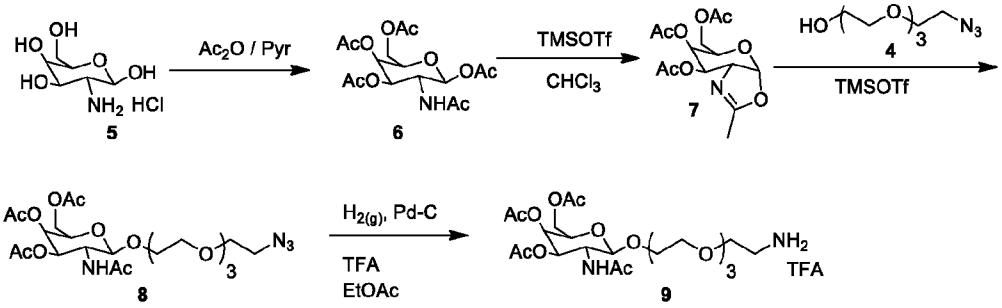
The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

## Example 1. Synthesis of conjugate 1

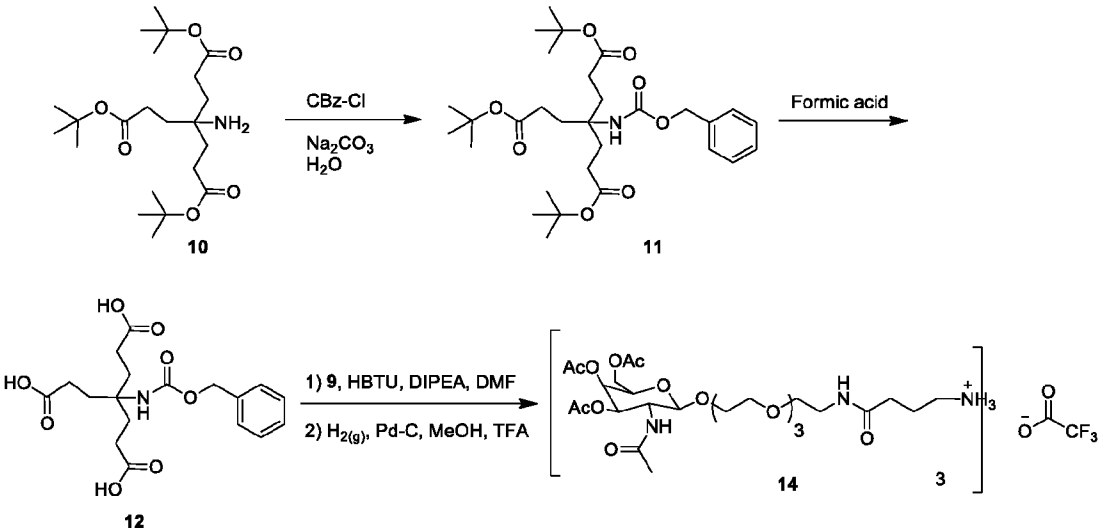
### Scheme 1.



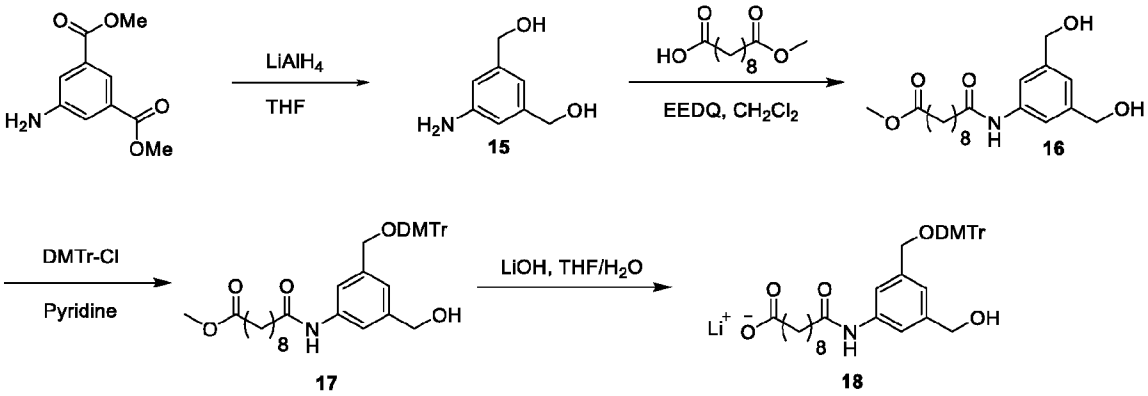

### Scheme 2.



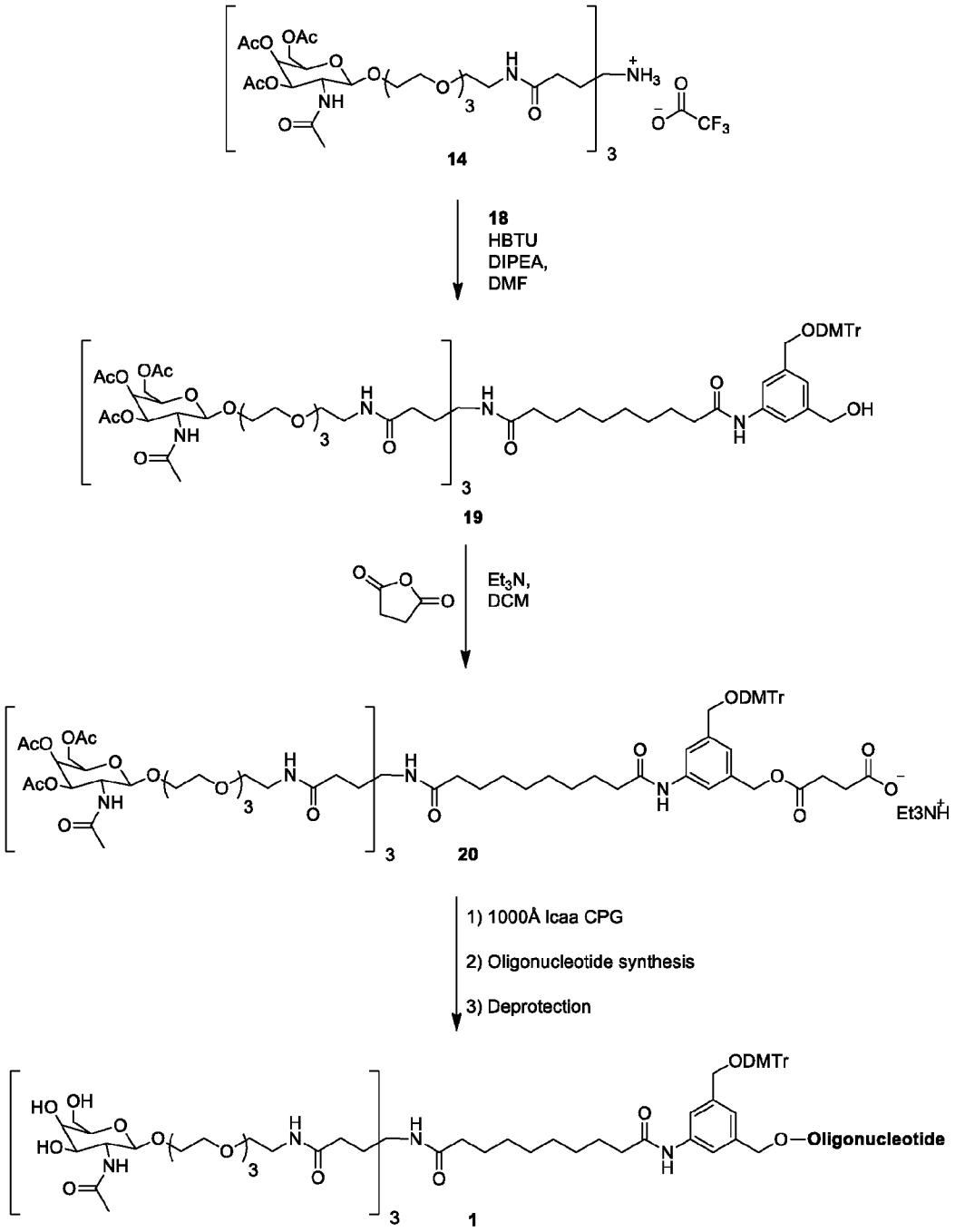

### Scheme 3.



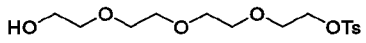

### Scheme 4.



**Scheme 5.**

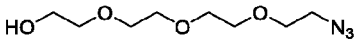



**Step 1. Preparation of 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate 3**



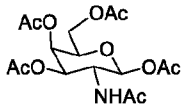
A solution of tetraethylene glycol (934 g, 4.8 mol) in THF (175mL) and aqueous NaOH (5M, 145 mL) was cooled (0°C) and treated with *p*-Toluensulfonyl chloride (91.4 g, 480 mmol) dissolved in THF (605 mL) and then stirred for two hours (0°C). The reaction mixture was diluted with water (3L) and extracted (3x 500mL) with $CH_2Cl_2$. The combined extracts were washed with water and brine then dried ($MgSO_4$), filtered and concentrated to afford 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate **3** (140 g, 84%) as a pale yellow oil. $R_f$ (0.57, 10% MeOH-$CH_2Cl_2$).

**Step 2. Preparation of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol 4**



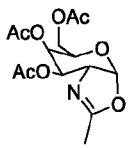

A solution of **3** (140 g, 403 mmol) in DMF (880 mL) was treated with sodium azide (131 g, 2.02 mol) and heated (45°C) overnight. A majority of the DMF was removed under reduced pressure and the residue was dissolved in $CH_2Cl_2$ (500 mL) and washed (3x 500 mL) with brine then dried ($MgSO_4$), filtered and concentrated. The residue was passed through a short bed of silica (5% MeOH-$CH_2Cl_2$) and concentrated to yield 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol **4** (65g, 74%) as a yellow oil. $R_f$ (0.56, 10% MeOH-$CH_2Cl_2$).

**Step 3. Preparation of peracetylated galactosamine 6**



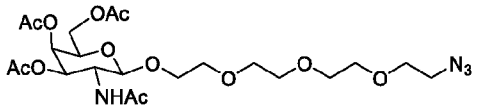

D-Galactosamine hydrochloride **5** (250 g, 1.16 mol) in pyridine (1.5 L) was treated with acetic anhydride (1.25 L, 13.2 mol) over 45 minutes. After stirring overnight the reaction mixture was divided into three 1 L portions. Each 1 L portion was poured into 3 L of ice water and mixed for one hour. After mixing the solids were filtered off, combined, frozen over liquid nitrogen and then lyophilized for five days to yield peracetylated galactosamine **6** (369.4 g, 82%) as a white solid. Rf (0.58, 10% MeOH-$CH_2Cl_2$).

**Step 4. Preparation of (3aR,5R,6R,7R,7aR)-5-(acetoxymethyl)-2-methyl-3a,6,7,7a-tetrahydro-5H-pyrano[3,2-d]oxazole-6,7-diyl diacetate 7**



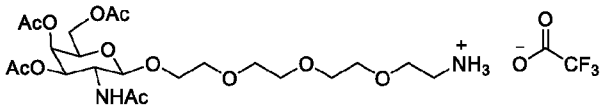
A solution of per-acetylated galactosamine **6** (8.45 g, 21.7 mmol) in $CHCl_3$ (320 mL) was treated dropwise with TMSOTf (4.32 mL, 23.9 mmol). After stirring (1.5 hr, 40°C) the reaction was quenched by the addition of triethylamine (5 mL) and concentrated to dryness to afford compound 7 as a pale yellow glass (7.2 g, Quant.). The product was used without further purification. Rf (0.59, 10% MeOH-$CH_2Cl_2$).

**Step 5. Preparation 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 8**



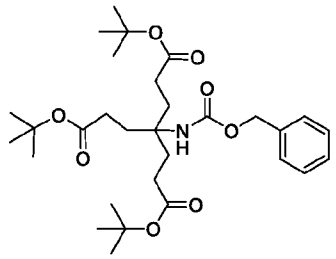

Compound 7 (7.2 g, 21.7 mmol) and 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethan-1-ol **4** (2.65 g, 15.2 mmol) were azeotroped (3x) from toluene (150 mL) to remove traces of water. The dried material was dissolved in 1,2-dichloroethane (150 mL), cooled (~5°C) and treated with TMSOTf (784 µL, 4.34 mmol). After stirring overnight the reaction was quenched by the addition of triethylamine (5 mL) and concentrated. The residue was purified by chromatography (1% → 5% MeOH-$CH_2Cl_2$) to afford **8** (7.12 g, 85%) as a brown oil. Rf (0.3, 10% MeOH-$CH_2Cl_2$).

**Step 6. Preparation of 2-(2-(2-(2-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethan-1-aminium 2,2,2-trifluoroacetate 9**



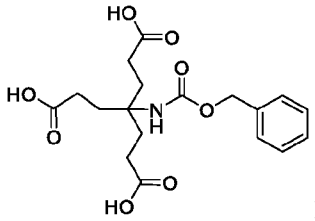

A solution of the azide **8** (7.12 g, 13 mmol) in EtOAc (150 mL) and trifluoroacetic acid (2 mL) was treated with palladium on charcoal (1.5 g, 10% w/w wet basis). The reaction mixture was then purged with hydrogen and stirred vigorously overnight. After purging with nitrogen, the mixture was filtered through Celite, rinsing with MeOH. The filtrate was concentrated and purified via chromatography (5% → 10% → 20% MeOH-$CH_2Cl_2$) to afford **9** (5.8 g, 72%) as a brown oil. Rf (0.34, 15% MeOH-$CH_2Cl_2$).

**Step 7. Preparation of di-tert-butyl 4-(((benzyloxy)carbonyl)amino)-4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate 11**



To a solution of di-tert-butyl 4-amino-4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate **10** (13.5 g, 33 mmol), 25% $Na_2CO_{3\,(aq)}$ (150 mL) and dichloromethane (300 mL) was added slowly benzyl chloroformate (14 mL, 98 mmol). The solution was stirred vigorously overnight (16h) at room temperature. Upon completion, additional dichloromethane (100 mL) was added and the dichloromethane layer was separated. The aqueous layer was extracted with dichloromethane (2 x 100 mL). The combine dichloromethane extracts were dried on magnesium sulfate, filtered and concentrated to dryness. The product **11** was isolated as a colorless oil that required no further purification (15.8 g, 88%). Rf (0.7, 1:1 EtOAc-Hexane).

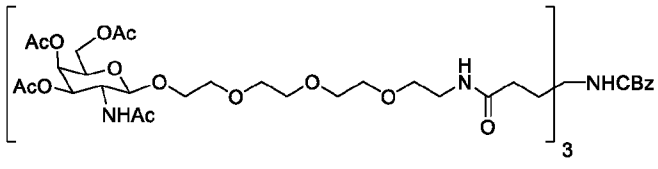
**Step 8. Preparation of 4-(((benzyloxy)carbonyl)amino)-4-(2-carboxyethyl)heptanedioic acid 12**



**12**

A solution of **11** (15.6 g, 28.8 mmol) in formic acid (50 mL) was stirred at room temperature for 2 hours. The solution was concentrated to dryness and dissolved in ethyl acetate (~25 mL). Upon standing, the product crystallized as a colorless solid. The solid was filtered, washed with ethyl acetate and air dried to afford **12** as a colorless solid (10.2 g, 93%). Rf (0.1, 10% MeOH-$CH_2Cl_2$).

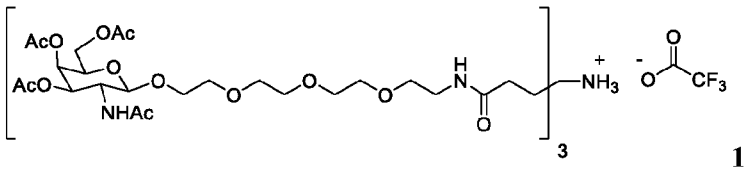
**Step 9. Preparation of compound 13**



**13**

A solution of **12** (793 mg, 2.08 mmol) and **9** (5.8 g, 9.36 mmol) in DMF (50mL) was treated with BOP (3.67 g, 8.32 mmol) then *N*,*N*-diisopropylethylamine (4.31 mL, 25 mmol). After stirring overnight the mixture was concentrated to dryness and subjected to chromatography (1% → 2% → 5% → 10% → 15% MeOH-$CH_2Cl_2$) to afford **13** (5.71 g [crude], >100% - contained coupling by-products that did not affect the next step). Rf (0.45, 10% MeOH-$CH_2Cl_2$).

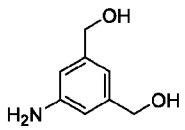
**Step 10. Preparation of compound 14**



**14**

Compound **13** (5.7 g) was dissolved in MeOH (150 mL) and TFA (1.5 mL) and treated with palladium on charcoal (1 g, 10% w/w wet basis). The reaction mixture was then purged with hydrogen and stirred vigorously overnight. After purging with nitrogen, the mixture was filtered through Celite, rinsing with MeOH. The filtrate was concentrated and purified via chromatography (5% → 10% → 20% MeOH-$CH_2Cl_2$) to afford **14** as a brown oil (2.15 g, 56% over two steps). Rf (0.32, 10% MeOH-$CH_2Cl_2$).

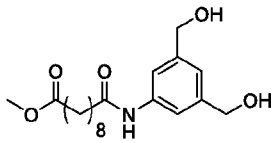
**Step 11. Preparation of (5-amino-1,3-phenylene)dimethanol 15**



A solution of dimethyl 5-aminoisophthalate (20.0 g, 96 mmol) in THF (350 mL) was added, dropwise, to a refluxing mixture of 3.75 eq $LiAlH_4$ (13.6 g, 358 mmol) in THF (440 mL) over one hour. The mixture was stirred at reflux for a further two hours, then cooled to room temperature and quenched by the careful addition of MeOH (27 mL) then water (40 mL). After stirring the quenched mixture for two hours it was filtered and concentrated to dryness. The

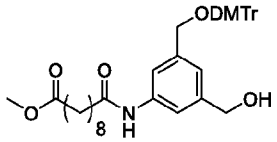
residue was recrystallized (2X) from EtOAc to afford **15** as brownish-yellow crystals (10.2 g, 70 %).

**Step 12. Preparation of methyl 10-((3,5-bis(hydroxymethyl)phenyl)amino)-10-oxodecanoate 16**



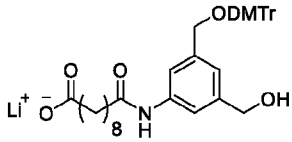
A solution of methyl sebacate (3.8 g, 17 mmol), **15** (2.5 g, 17 mmol) and EEDQ (8.1 g, 33 mmol) in 2:1 dichloromethane / methanol (200 mL) was stirred at room temperature for 2 hours. Upon completion the solution was concentrated to dryness. The solid obtained was triturated with dichloromethane (50 mL) and filtered. The solid was rinsed with cold dichloromethane and air dried to afford **16** as a colorless solid (4.3 g, 72%). Rf (0.33, EtOAc).

**Step 13. Preparation of methyl 10-((3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(hydroxymethyl)phenyl)amino)-10-oxodecanoate 17**



To a solution of **16** (4.3 g, 12 mmol) in pyridine (50 mL) was added 4,4'-(chloro(phenyl)methylene)bis(methoxybenzene) (4.1 g, 12 mmol). The solution was stirred under nitrogen overnight at room temperature. Upon completion the solution was concentrated to dryness and the residue was purified by column chromatography (0.5% → 0.75% → 1% → 1.5% MeOH-$CH_2Cl_2$) to afford **17** as a yellow solid (2.9 g, 35%). Rf (0.6, 10% MeOH-$CH_2Cl_2$).

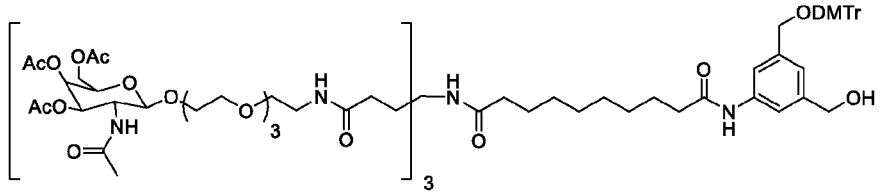
**Step 14. Preparation of lithium 10-((3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(hydroxymethyl)phenyl)amino)-10-oxodecanoate 18**



To a solution of **17** (2.9 g, 4.3 mmol) in THF (60 mL) was added water (15 mL) and lithium hydroxide (112 mg, 4.7 mmol). The solution was stirred overnight at room temperature. Upon completion the solution was concentrated to remove the THF. The remaining aqueous

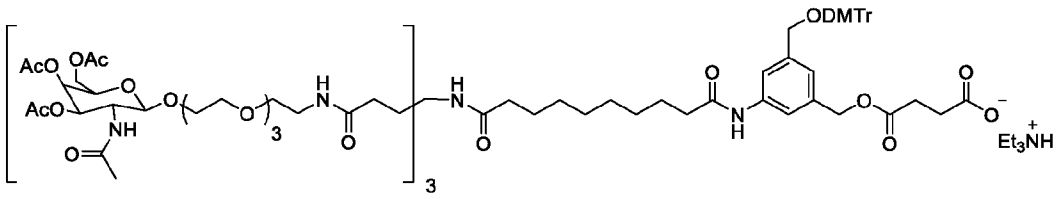
solution was flash frozen on liquid nitrogen and lyophilized overnight to afford a colorless solid (2.9 g, quant.). Rf (0.3, 10% MeOH-$CH_2Cl_2$).

**Step 15. Preparation of compound 19**



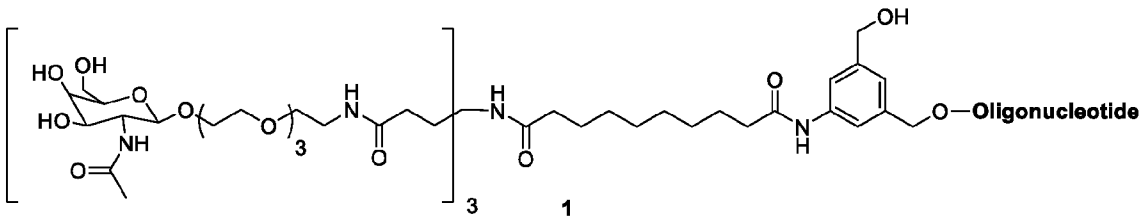

To a solution 14 (454 mg, 0.67 mmol), 18 (1.25 g, 0.67 mmol) and HBTU (381 mg, 1.0 mmol) in anhydrous DMF (25 mL) was added *N,N*-diisopropylethylamine (0.35 mL, 2.0 mmol). The solution was stirred overnight at room temperature. Upon completion, the solution was poured into ethyl acetate (250 mL) and washed with brine (3 x 200 mL). The ethyl acetate layer was dried on magnesium sulfate, filtered and concentration to dryness. Purification by column chromatography (5% → 7.5% → 10% → 15% MeOH in $CH_2Cl_2$) afforded **19** as a pale orange foam (1.5 g, 94%). Rf (0.25, 10% MeOH-$CH_2Cl_2$).

**Step 16. Preparation of compound 20**




A solution of compound **19** (1.5 g, 0.6 mmol), succinic anhydride (120 mg, 1.2 mmol), DMAP (220 mg, 1.8 mmol) and trimethylamine (250 µL, 1.8 mmol) in anhydrous $CH_2Cl_2$ (50 mL) was stirred overnight at room temperature. Upon completion, the solution was concentrated to dryness and filtered through a short plug of silica (100% $CH_2Cl_2$ → 15% MeOH in $CH_2Cl_2$) to afford the product **20** as a light beige foam (1.1 g, 70%). Mass *m/z* (ES-TOF MS) 727.7 [M + 3H - DMTr]$^+$, 1091.1 [M + 2H - DMTr]. $^1$H NMR (400 MHz, $CDCl_3$) δ 8.92 (br s, 1H), 7.78 (s, 1H), 7.49-7.47 (m, 3H), 7.41 (br s, 1H), 7.38-7.34 (m, 5H), 7.32-7.26 (m, 4H), 7.24-7.08 (br s, 3H), 7.08 (s, 1H), 6.90-6.80 (m, 7H), 5.31 (d, 3H, $J$ = 2.7Hz), 5.12 (s, 2H), 5.06 (dd, 3H, $J$ = 11.2, 3.2 Hz), 4.78 (d, 3H, $J$ = 8.5 Hz), 4.24-4.08 (m, 12H), 3.95-3.88 (m, 7H), 3.85-3.76 (m, 4H), 3.78 (s, 6H), 3.68-3.56 (m, 34H), 3.54-3.44 (m, 8H), 3.41-3.33 (m, 6H), 2.70-2.60 (m, 4H), 2.52-2.30 (m, 30H), 2.24-2.16 (m, 8H), 2.14 (s, 9H), 2.04 (s, 9H), 2.02-1.96 (m, 6H), 1.98 (s, 9H), 1.96 (s, 9H), 1.74-1.52 (m, 4H), 1.36-1.24 (m, 12H).

**Step 17. Preparation of conjugate 1**

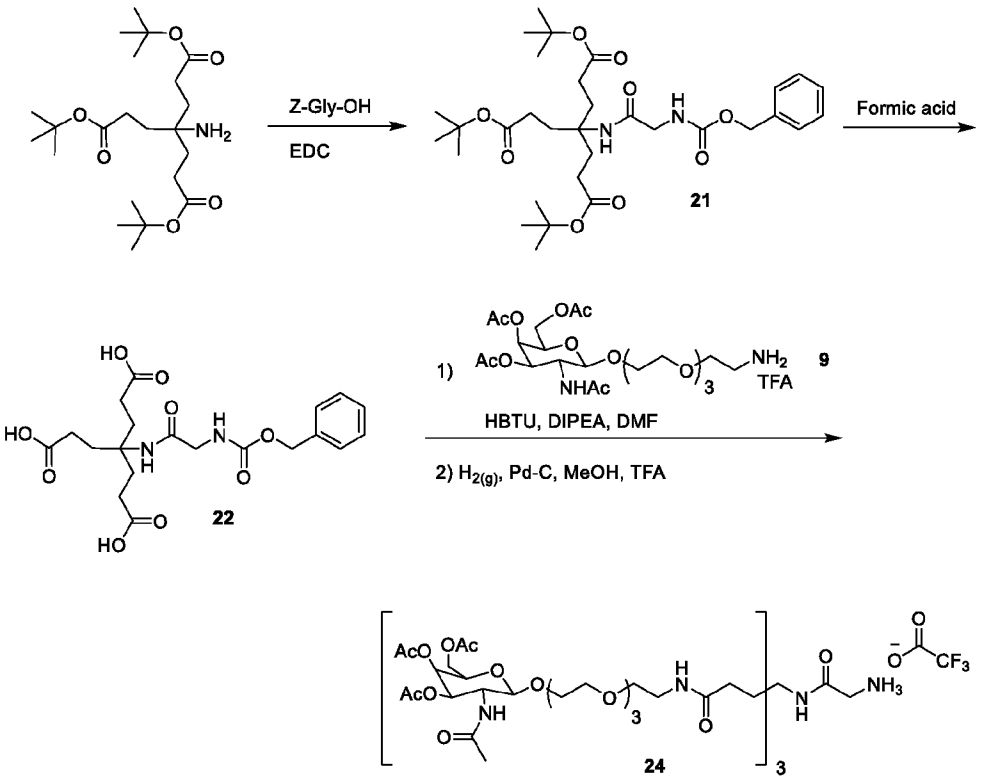



1

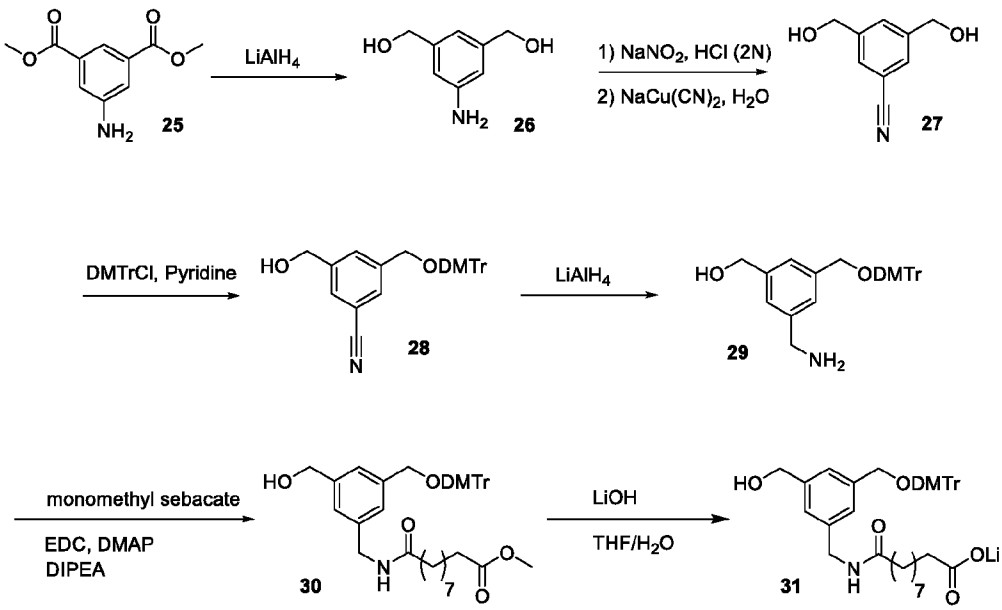
The succinate **20** was loaded onto 1000Å LCAA (long chain aminoalkyl) CPG (control pore glass) using standard amide coupling chemistry. A solution of diisopropylcarbodiimide (52.6 μmol), N-hydroxy succinimide (0.3 mg, 2.6 μmol) and pyridine (10 μL) in anhydrous acetonitrile (0.3 mL) was added to **20** (20.6 mg, 8 μmol) in anhydrous dichloromethane (0.2 mL). This mixture was added to LCAA CPG (183 mg). The suspension was gently mixed overnight at room temperature. Upon disappearance of **20** (HPLC), the reaction mixture was filtered and the CPG was washed with 1 mL of each dichloromethane, acetonitrile, a solution of 5% acetic anhydride / 5% N-methylimidazole / 5% pyridine in THF, then THF, acetonitrile and dichloromethane. The CPG was then dried overnight under high vacuum. Loading was determined by standard DMTr assay by UV/Vis (504 nm) to be 25 μmol/g. The resulting GalNAc loaded CPG solid support was employed in automated oligonucleotide synthesis using standard procedures. Nucleotide deprotection followed by removal from the solid support (with concurrent galactosamine acetate deprotection) afforded the GalNAc-oligonucleotide conjugate **1** as a representative example.

## Example 2: Synthesis of conjugate 34

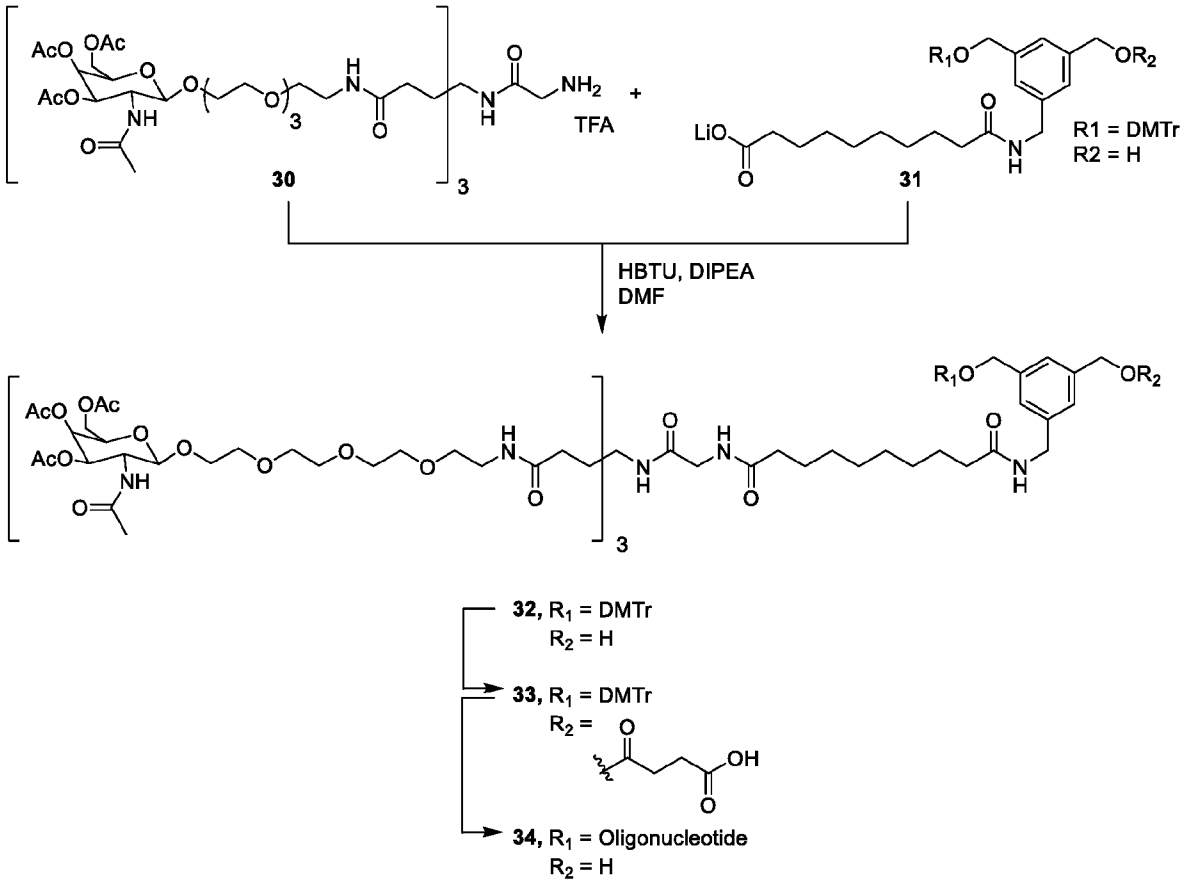
**Scheme 6.**



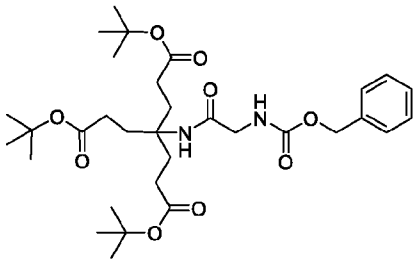

**Scheme 7.**



**Scheme 8.**



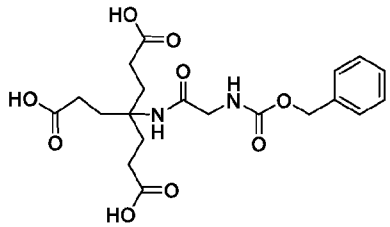

**Step 1. Preparation of di-tert-butyl 4-(2-(((benzyloxy)carbonyl)amino)acetamido)-4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate 21**



A solution of di-tert-butyl 4-amino-4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate (25 g, 60 mmol) and Z-glycine (18.9 g, 90.2 mmol,) in $CH_2Cl_2$ (300 mL) was treated successively with EDC (23 g, 120 mmol), Diisopropylethylamine (32 mL, 180 mmol) and DMAP (Cat. 17 mg). After stirring (16h) the reaction mixture was poured into $NaHCO_3$ (Sat. Aq.), extracted with $CH_2Cl_2$, washed with brine, dried ($MgSO_4$), filtered and concentrated to afford di-tert-butyl 4-(2-(((benzyloxy)carbonyl)amino)acetamido)-4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate **21** as an

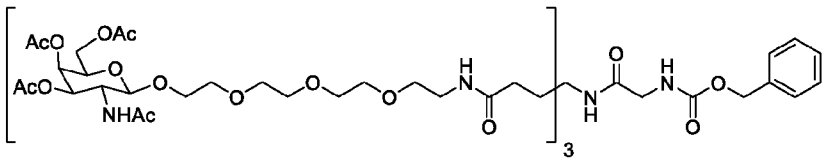
amorphous solid and was used without further processing (36 g, quant.). Rf (0.85, 10% MeOH-$CH_2Cl_2$).

**Step 2. Preparation of 4-(2-(((benzyloxy)carbonyl)amino)acetamido)-4-(2-carboxyethyl)heptanedioic acid 22**



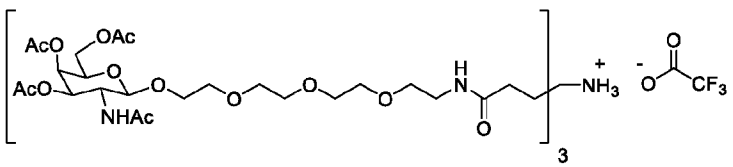
A solution of di-tert-butyl 4-(2-(((benzyloxy)carbonyl)amino)acetamido)-4-(3-(tert-butoxy)-3-oxopropyl)heptanedioate **21** (59.3mmol, 36g) was stirred in neat formic acid (150mL) for 72 hours. Upon completion, the formic acid was removed under reduced pressure and the crude solid was dried overnight on high-vacuum to yield **22** as a colorless solid (15.9 g, 61%). Rf (0.15, 10% MeOH-$CH_2Cl_2$).

**Step 3. Preparation of compound 23**



A solution of **22** (6.2 g, 14.1 mmol) and 2-(2-(2-(2-(((2R,3R,4R,5R,6R)-3-acetamido-4,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2H-pyran-2-yl)oxy)ethoxy)ethoxy)ethoxy)ethan-1-aminium 2,2,2-trifluoroacetate (35 g, 56.5 mmol) in DMF (250mL) was treated with BOP (25 g, 56.5 mmol) then *N*,*N*-diisopropylethylamine (29 mL, 170 mmol). After stirring overnight the mixture was concentrated to dryness and subjected to chromatography (100% $CH_2Cl_2$ to 15% MeOH-$CH_2Cl_2$) to afford compound **23** (24.6 g, 89%). Rf (0.55, 15% MeOH-$CH_2Cl_2$).

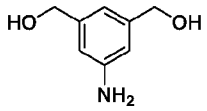
**Step 4. Preparation of compound 24**



Compound **23** (24.6 g) was dissolved in MeOH (200 mL) and TFA (1.5 mL) and purged with nitrogen. Palladium on charcoal (1 g, 10% w/w wet basis) was added and then the reaction

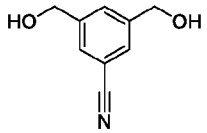
mixture was purged with hydrogen and stirred vigorously overnight. Upon completion, the reaction was purged with nitrogen, filtered through Celite and rinsed with MeOH. The filtrate was concentrated and purified by column chromatography on silica gel 60 (gradient: 5% → 10% → 20% MeOH-$CH_2Cl_2$) to afford **24** as a pale brown viscous oil (23 g). Rf (0.32, 10% MeOH-$CH_2Cl_2$).

**Step 5. Preparation of (5-amino-1,3-phenylene)dimethanol 26**



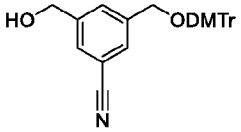

A suspension of lithium aluminum hydride (13.6 g, 358 mmol) in anhydrous tetrahydrofuran (450 mL) was brought to reflux under a nitrogen atmosphere and treated, dropwise, with a solution of dimethyl-5-aminoisophthalte **25** (20 g, 96 mmol) in anhydrous tetrahydrofuran (350 mL). After the addition was complete the mixture was heated to reflux for an additional 2 hours. Upon completion, the solution was cooled to room temperature and quenched by the slow addition of MeOH (27 mL) then water (40 mL). After stirring for 2 hours the mixture was filtered, concentrated and recrystallized from EtOAc to yield (5-amino-1,3-phenylene)dimethanol **26** as off-white crystals (10.2 g, 70%). Rf 0.5 (15% MeOH-$CH_2Cl_2$).

**Step 6. Preparation of 3,5-bis(hydroxymethyl)benzonitrile 27**



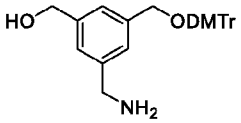

A solution of **26** (5 g, 33 mmol) in 2N hydrochloric acid (100 mL) was cooled to $0^0$C and treated with a cold solution of sodium nitrite (3.53 g, 36mmol) in water (50 mL). The reaction mixture was maintained at a temperature $\leq 5^0$C for 30min then treated with a solution of copper(I) cyanide (3.19 g, 35.6mmol) and sodium cyanide (3.53 g, 72mmol) in water (50 mL) in a single portion. After stirring overnight at room temperature the mixture was filtered, extracted with dichloromethane (3 x 100 mL), concentrated and used without further purification. The diol, 3,5-bis(hydroxymethyl)benzonitrile **27** was obtained as a yellow solid (2.19 g, 41%). Rf 0.75 (15% MeOH-$CH_2Cl_2$).

**Step 7. Preparation of 3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(hydroxymethyl)benzonitrile 28**



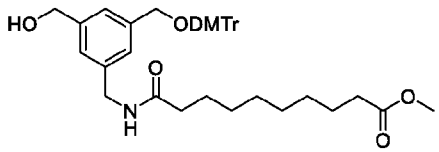

A solution of 3,5-bis(hydroxymethyl)benzonitrile **27** (538 mg, 3.3 mmol) in pyridine (14 mL) was treated with 4,4′-Dimethoxytrityl chloride (1.17 g, 3.46 mmol) and stirred overnight at room temperature. Once complete, the mixture was concentrated and dispersed in diethyl ether (25 mL), filtered and concentrated. The crude product was purified by column chromatography of silica gel 60 (gradient: 10% to 50% EtOAc-Hexane) to yield the **28** as a yellow solid (725 mg, 47%). Rf 0.5 (1:1 EtOAc-hexane).

**Step 8. Preparation of (3-(aminomethyl)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)phenyl)methanol 29**




A solution of the **28** (100 mg, 0.22 mmol) in methyl tetrahydrofuran (5 mL) was cooled to 0°C and treated slowly with lithium aluminum hydride (0.64 mmol = 0.28mL of a 2.3M solution in MeTHF). After stirring for one hour the reaction was quenched by the addition of methanol (1 mL) then water (0.3 mL) and stirred for 30min. The mixture was filtered and concentrated, to yield (3-(aminomethyl)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)phenyl)methanol **29** (78 mg, 77%). Rf 0.15 (10% MeOH-$CH_2Cl_2$).

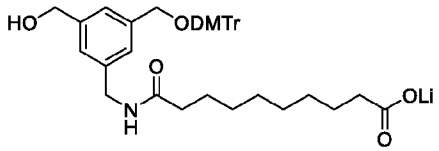
**Step 9. Preparation of methyl 10-((3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(hydroxymethyl)benzyl)amino)-10-oxodecanoate 30**




A solution of (3-(aminomethyl)-5-((bis(4-methoxyphenyl)(phenyl)methoxy)-methyl)phenyl)methanol **29** (78 mg, 0.17 mmol) and monomethyl sebacate (38 mg, 0.17 mmol,) in dichloromethane (5 mL) were treated successively with EDC (48 mg, 0.25 mmol), DMAP (cat., 5 mg) and diisopropylethylamine (57 µL, 0.33 mmol). After stirring (3.5 hr) the reaction

mixture was poured into saturated sodium bicarbonate solution (50 mL). The sodium bicarbonate solution was extracted with dichloromethane (3 x 50 mL), washed with brine (50 mL), dried on magnesium sulfate, filtered and concentrated to dryness. The crude material was purified by column chromatography on silica gel 60 (gradient: 2% to 5% MeOH-$CH_2Cl_2$) to afford methyl 10-((3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(hydroxymethyl)benzyl)amino)-10-oxodecanoate **30** as a yellow oil (57 mg, 53%). Rf 0.45 (10% MeOH-$CH_2Cl_2$).

**Step 10. Preparation of lithium 10-((3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(hydroxymethyl)benzyl)amino)-10-oxodecanoate 31**

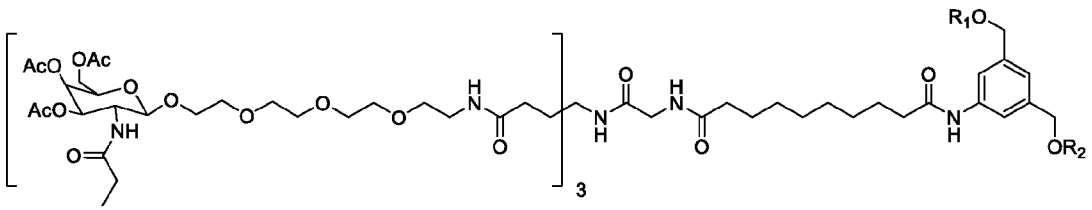



Compound **30** (188 mg, 0.28 mmol) was dissolved in tetrahydrofuran (5 mL) and treated with a solution of LiOH (7mg, 0.30 mmol) in water (1 mL). Upon completion, the tetrahydrofuran was removed *in vacuo* and the remaining aqueous mixture was frozen and lyophilized to afford lithium 10-((3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-(hydroxymethyl)benzyl)amino)-10-oxodecanoate **31** as a colorless solid (180 mg, 99%). Rf 0.45 (10% MeOH-$CH_2Cl_2$).

**Step 11. Preparation of compounds 32, 33, and 34**

Compounds **32**, **33** and **34** were prepared according to same procedure used to synthesize compounds **19**, **20**, and **1** respectfully.

**Example 3. Synthesis of conjugate 36**



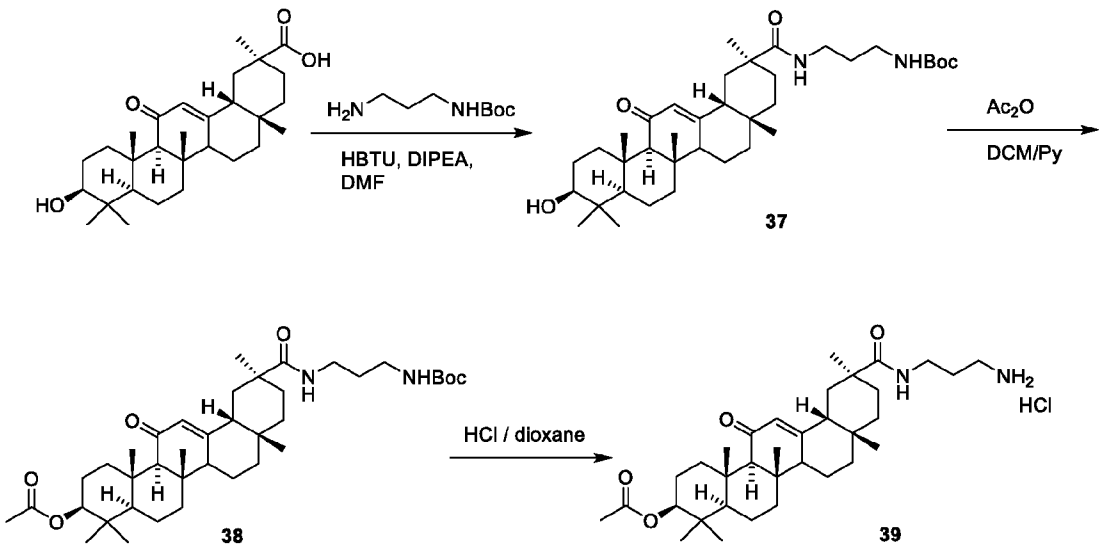

**36**, $R_1$ = Oligonucleotide
$R_2$ = H

**Step 1. Preparation of conjugate 36**

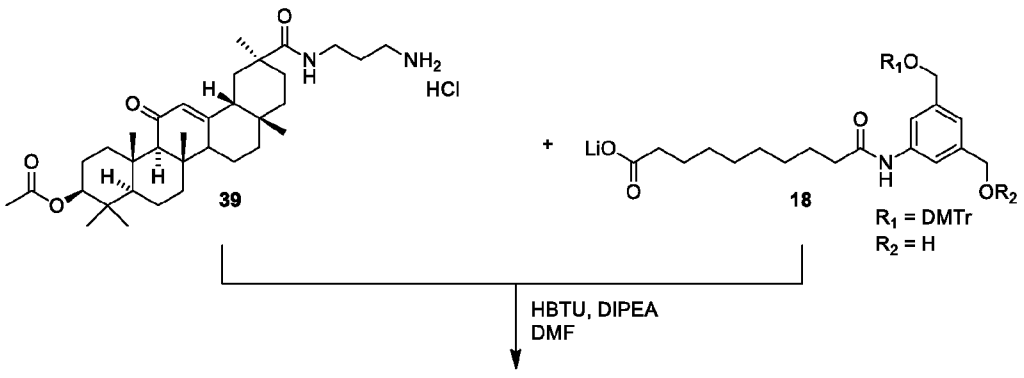
Conjugate **36** was prepared using identical procedures as used to synthesize compound **34** and all corresponding intermediates. The only exception being the synthesis of compound **6** where propanoic anhydride was used in place of acetic anhydride.

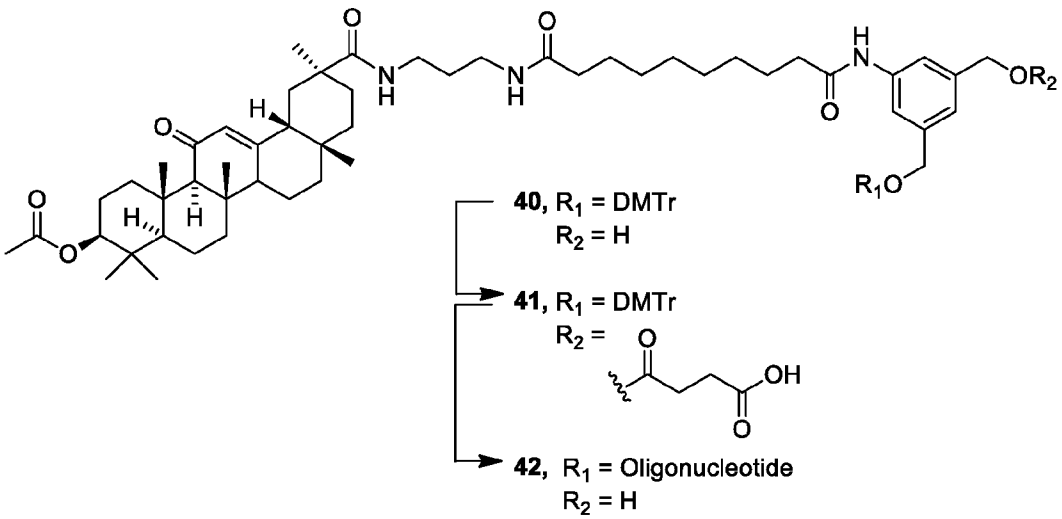
**Example 4. Synthesis of conjugate 42**

**Scheme 9.**

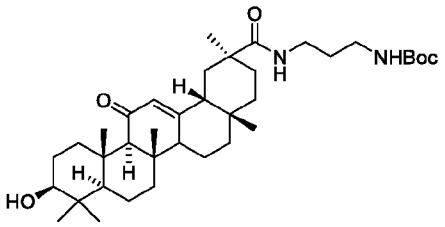



**Scheme 10.**



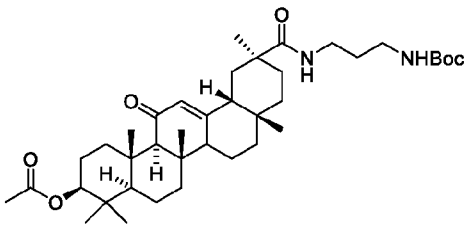



**Step 1. Preparation of compound 37**




A solution of 18β-glycyrrhetinic acid (2.5 g, 5.3 mmol), tert-butyl (3-aminopropyl)carbamate (1.1 g, 6.4 mmol) and HBTU (3.0 g, 8.0 mmol) in *N*,*N*-dimethylformamide (20 mL) was added diisopropylethylamine (2.75 mL, 15.9 mmol). The solution was stirred overnight at room temperature. Upon completion, the solution was concentrated in vacuo to dryness. The residue was purified by column chromatography on silica gel 60 (gradient: 2% to 5% MeOH/$CH_2Cl_2$) to afford the product as a colorless solid (2.1 g, 63%).

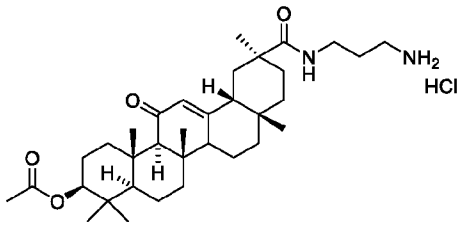
**Step 2. Preparation of compound 38**




To a solution of **37** (2.1 g, 3.3 mmol) and triethylamine (3.5 mL, 10 mmol) in dichloromethane (25 mL) was added acetic anhydride (850 µL, 5.3 mmol) and DMAP (5 mg). The solution was stirred overnight at room temperature. Upon completion, the solution was concentrated to dryness and dissolved in ethyl acetate (100 mL), washed with water (100 mL),

dried on magnesium sulfate, filtered and concentrated to dryness to afford a pale brown foam (1.9 g, 85%).

**Step 3. Preparation of compound 39**



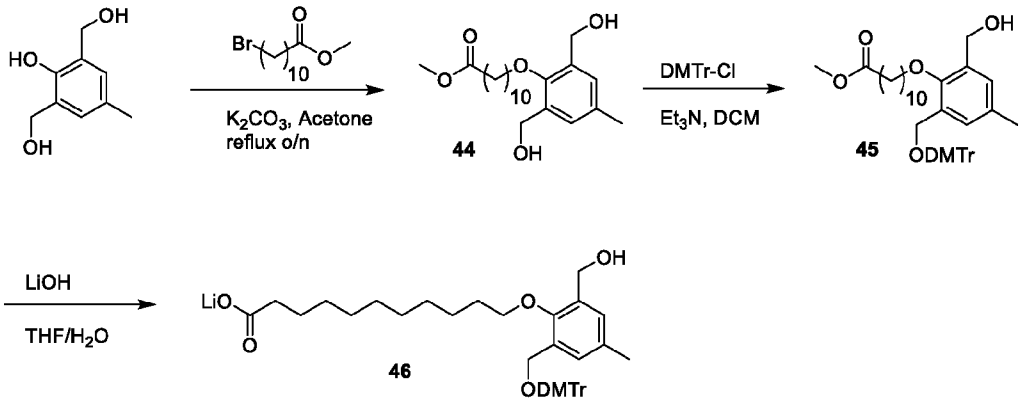
To a solution of 38 (1.5 g, 2.3 mmol) in anhydrous dioxane (25 mL) was added 2M Hydrogen chloride in dioxane (25 mL). The solution was stirred overnight at room temperature then concentrated in vacuo to dryness to afford a light brown solid (1.3 g, 96%).

**Step 4. Preparation of compounds 40, 41 and 42**

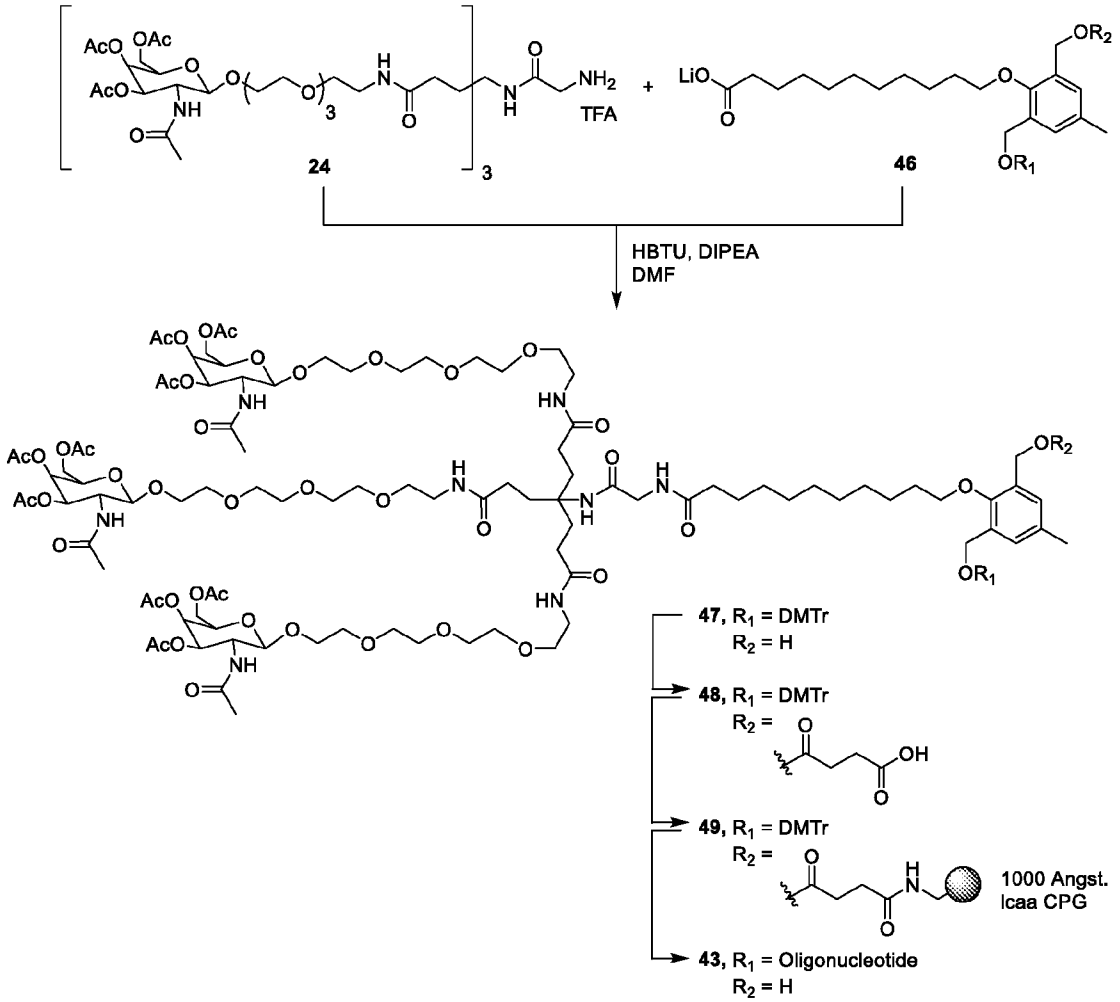
Compounds **40**, **41** and **42** were prepared according to the same procedure used to synthesize compounds **19**, **20**, and **1** respectfully.

**Example 5. Synthesis of Conjugate 43**

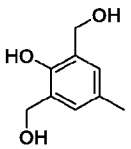
**Scheme 11.**



**Scheme 12.**



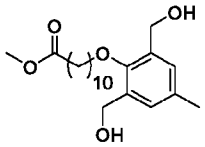

**Step 1. Preparation of methyl 11-(2,6-bis(hydroxymethyl)-4-methylphenoxy)undecanoate 44**



To a solution of 2,6-bis(hydroxymethyl)-p-cresol (2.7 g, 16.3 mmol), methyl 11-bromoundecanoate (5.0 g, 17.9 mmol) and potassium carbonate (4.5 g, 32.6 mmol) in acetone (100 mL) was refluxed for 16 hours. Upon completion the solution was concentrated *in vacuo* to dryness, suspended in ethyl acetate (150 mL) and washed with water (2 x 100 mL) and brine (100 mL). The ethyl acetate layer was dried on magnesium sulfate, filtered and concentrated *in vacuo* to dryness. The residue was purified by column chromatography on silica gel 60 (gradient

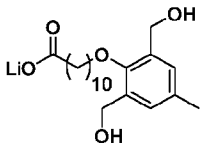
100 % Hex → 50% EtOAc/Hex) to afford methyl 11-(2,6-bis(hydroxymethyl)-4-methylphenoxy)undecanoate **44** as a colorless oil (1.6 g, 27%).

**Step 2. Preparation of methyl 11-(2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-6-(hydroxymethyl)-4-methylphenoxy)undecanoate 45**



To a solution of methyl 11-(2,6-bis(hydroxymethyl)-4-methylphenoxy)undecanoate **44** (1.5 g, 4.1 mmol) in anhydrous pyridine (20 mL) was added 4,4′-Dimethoxytrityl chloride (1.4 g, 4.1 mmol). The solution was stirred overnight at room temperature. Upon completion the solution was concentrated *in vacuo* to dryness and purified by column chromatography on silica gel 60 (0.5 to 1% MeOH in $CH_2Cl_2$) to afford Methyl 11-(2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-6-(hydroxymethyl)-4-methylphenoxy)undecanoate **45** as a pale yellow solid (1.1 g, 40%).

**Step 3. Preparation of lithium 11-(2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-6-(hydroxymethyl)-4-methylphenoxy)undecanoate 46**



To a solution of Methyl 11-(2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-6-(hydroxymethyl)-4-methylphenoxy)undecanoate **45** (1.1 g, 1.7 mmol) in anhydrous tetrahydrofuran (40 mL) and water (10 mL) was added lithium hydroxide (44 mg, 1.8 mmol). The solution was concentrated *in vacuo* to remove all tetrahydrofuran. The remaining aqueous solution was flash frozen on liquid nitrogen then lyophilized overnight to afford lithium 11-(2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-6-(hydroxymethyl)-4-methylphenoxy)undecanoate **46** as a pale pink solid (1.1 g, 94%).

**Step 4. Preparation of Compound 47**

A solution of **10** (1.33 g, 0.66 mmol), **46** (0.5 g, 0.73 mmol), HBTU (400 mg, 1 mmol) in *N*,*N*-dimethylformamide (25 mL) was added diisopropylethylamine (0.35 mL, 2 mmol). The solution was stirred overnight (18 hours) at room temperature. Upon completion, the solvent was remove in vacuo and the residue was purified by column chromatography on silica gel (gradient:

100% $CH_2Cl_2$ - 5% - 10% - 15% MeOH in $CH_2Cl_2$) to afford **47** as a colorless solid (710 mg, 41%).

**Step 5. Preparation of Compound 48**

To a solution of **47** (0.71 g, 0.3 mmol), triethylamine (0.4 mL, 3.0 mmol) and polystyrene-DMAP (3 mmol/g loading, 200 mg, 0.6 mmol) in dichloromethane (15 mL) was added succinic anhydride (60 mg, 0.6 mmol). The solution was stirred overnight at room temperature and upon completion filtered and concentrated *in vacuo* to dryness. The residue was purified by column chromatography on silica gel 60 (gradient 5% to 20% MeOH in $CH_2Cl_2$) to afford the **48** as a pale yellow solid (570 mg, 70%). $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 7.91 (m, 1H), 7.86-7.76 (m, 6H), 7.45-7.40 (m, 2H), 7.36-7.14 (m, 10H), 7.10 (s, 1H), 6.91 (d, $J$ = 8.9 Hz, 4H), 5.21 (d, J = 3.3 Hz, 3H), 5.01 (s, 2H), 4.97 (dd, J = 11.2, 3.4 Hz, 3H), 4.56 (d, J = 8.5 Hz, 3H), 4.06-3.98 (m, 11H), 3.93-3.84 (m, 3H), 3.81-3.72 (m, 3H), 3.74 (s, 6H), 3.65-3.46 (m, 38H), 3.40-3.35 (m, 6H), 3.20-3.16 (m, 6H), 2.56-2.44 (m, 4H), 2.33 (s, 3H), 2.15-2.08 (m, 2H), 2.10 (s, 9H), 2.04-1.96 (m, 6H), 1.89 (s, 9H), 1.82-1.76 (m, 4H), 1.77 (s, 9H), 1.54-1.34 (m, 4H), 1.28-1.10 (m, 12H),

**Step 6. Preparation of compound 49**

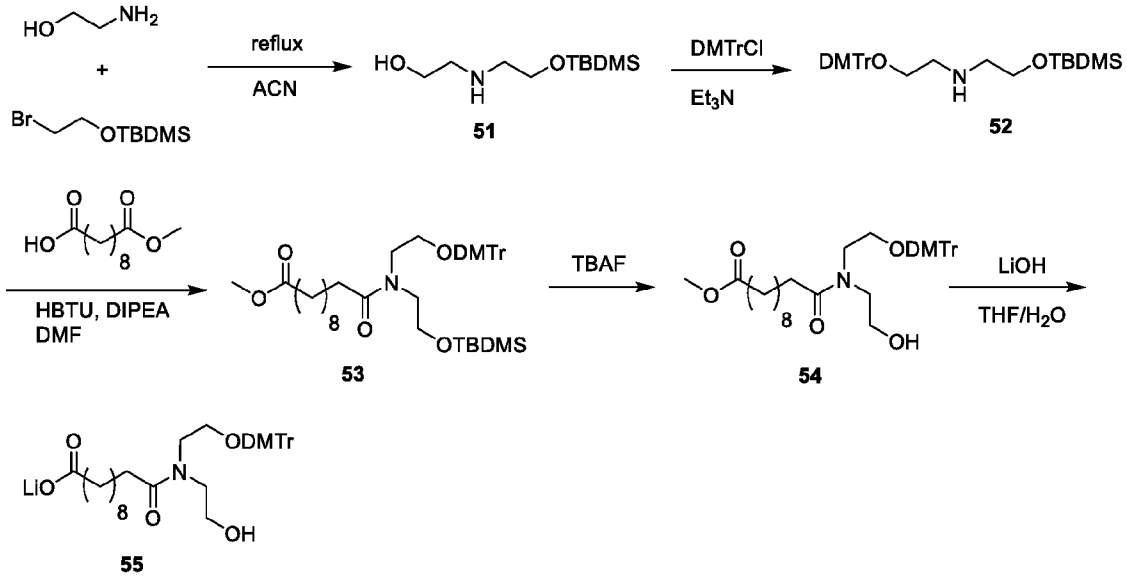
To a solution of **48** (100 mg, 40 μmol), *N*-Hydroxysuccinimide (30 mg/mL soln in acetonitrile, 50 μL, 13 μmol), *N*,*N*'-Diisopropylcarbodiimide (40 μL, 264 μmol) and pyridine (50 μL) in dichloromethane (2 mL) and acetonitrile (3 mL) was added 1000 Å lcaa CPG (prime synthesis, 920 mg). The solution was stirred overnight at room temperature on an orbital shaker. TLC analysis of the reaction solution showed only partial consumption of the activated *N*-Hydroxysuccinic ester so additional CPG (500 mg) was added. The solution was stirred again overnight. Upon completion, the CPG was filtered and washed with dichloromethane (25 mL), acetonitrile (25 mL) and tetrahydrofuran (25 mL). The unreacted amine residues on the CPG were acetylated (capped) by adding a 1:1 solution of acetic anhydride in acetonitrile (3 mL) and 10% N-methylimidazole / 10% pyridine in tetrahydrofuran (3 mL). The suspension was left for 2 hours then filtered and rinsed with equal parts tetrahydrofuran (25 mL), acetonitrile (25 mL) and dichloromethane (25 mL). The loaded CPG **49** was dried under high vacuum overnight. The ligand loading efficiency was determined to be 22 μmole/g using a standard DMT loading assay (3% trichloroacetic acid in $CH_2Cl_2$, UV-VIS, $A_{504}$).

**Step 7. Preparation of conjugate 43**

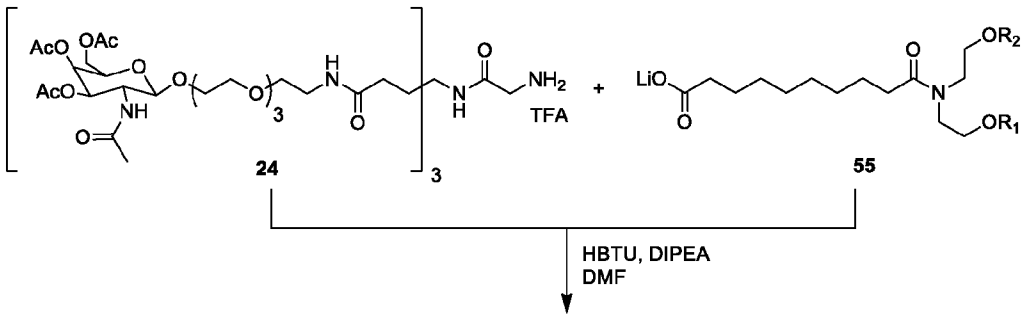
The resulting GalNAc loaded CPG solid support **49** was employed in automated oligonucleotide synthesis using standard procedures. Nucleotide deprotection followed by removal from the solid support (with concurrent galactosamine acetate deprotection) afforded a GalNAc-oligonucleotide conjugate **43**.

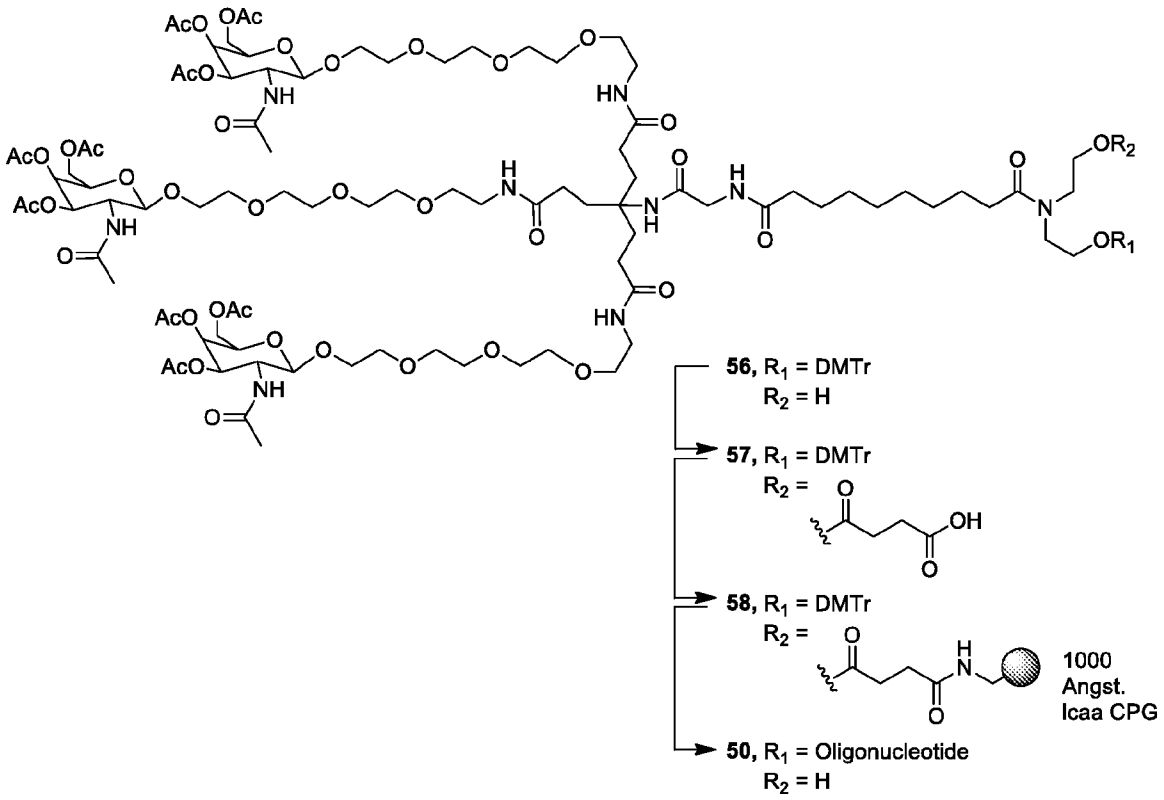
**Example 6. Synthesis of Conjugate 50**

**Scheme 13.**

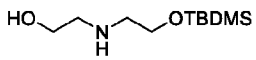



**Scheme 14.**



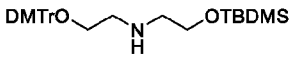



**Step 1. Preparation of 2-((2-((tert-butyldimethylsilyl)oxy)ethyl)amino)ethan-1-ol 51**




A solution of ethanolamine (77 mL, 1.25 mol) and (2-bromoethoxy)-*tert*-butyl dimethylsilane (15 g, 62.7 mmol) in anhydrous acetonitrile (200 mL) was refluxed for 3 hours. Upon completion the reaction was cooled to room temperature, diluted with water (400 mL) and extracted with ethyl acetate (3 x 150 mL). The combined ethyl acetate extracts were dried on magnesium sulfate, filtered and concentrated in vacuo to dryness. The residue was purified by filtration through a pad of silica first with 50% ethyl acetate/hexanes then 50% MeOH/EtOAc to afford **51** as a pale yellow oil (14 g, 100%).

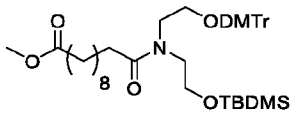
**Step 2. Preparation of 2-(bis(4-methoxyphenyl)(phenyl)methoxy)-N-(2-((tert-butyldimethylsilyl)oxy)ethyl)ethan-1-amine 52**




To a solution of 2-((2-((tert-butyldimethylsilyl)oxy)ethyl)amino)ethan-1-ol **51** (14 g, 64 mmol) and triethylamine (17.5 mL, 128 mmol) in anhydrous dichloromethane (250 mL) was added 4,4′-Dimethoxytrityl chloride (24 g, 70 mmol). The solution was stirred overnight at room

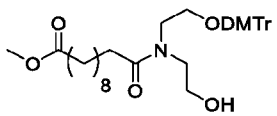
temperature then concentrated in vacuo to dryness. The residue was dissolved in ethyl acetate (300 mL) and washed with water (250 mL) and brine (250 mL). The ethyl acetate was dried on magnesium sulfate, filtered and concentrated in vacuo to dryness. Purification by column chromatography on silica gel 60 (1% to 5% MeOH in $CH_2Cl_2$) afforded **52** as a pale yellow viscous oil (13 g, 39%).

**Step 3. Preparation of methyl 10-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(2-((tert-butyldimethylsilyl)oxy)ethyl)amino)-10-oxodecanoate 53**



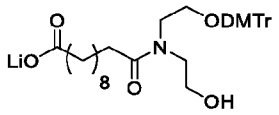

A solution of 2-(bis(4-methoxyphenyl)(phenyl)methoxy)-N-(2-((tert-butyldimethylsilyl)oxy)ethyl)ethan-1-amine **52** (5.4 g, 10.3 mmol), monomethyl sebacate (2.2 g, 10.3 g), HBTU (4.9 g, 12.9 mmol), DIPEA (5.3 mL, 30.9 mmol) in *N,N*-dimethylformamide (100 mL) was stirred for 3 hours at room temperature. Upon completion, the solution was poured into water (400 mL) and extracted with ethyl acetate (1 x 500 mL). The ethyl acetate extract was washed with brine (2 x 250 mL), dried on magnesium sulfate, filtered and concentrated in vacuo to dryness. Purification by column chromatography on silica gel 60 (10% to 25% ethyl acetate in hexanes) afforded **53** as a viscous yellow oil (6.5 g, 87%).

**Step 4. Preparation of methyl 10-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(2-hydroxyethyl)amino)-10-oxodecanoate 54**




To a solution of methyl 10-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(2-((tert-butyldimethylsilyl)oxy)ethyl)amino)-10-oxodecanoate **53** (2.0 g, 2.8 mmol) and triethylamine (1 mL) in anhydrous tetrahydrofuran (20 mL) was added TBAF (1M in THF, 3.4 mL, 3.3 mmol). The solution was stirred for 6h, but only partial conversion observed by TLC (5% MeOH in $CH_2Cl_2$). Additional 1.7 mL TBAF added and the solution was stirred overnight at room temperature. Upon completion, the solution was concentrated in vacuo and purified by column chromatography on silica gel 60 (10% to 50% EtOAc in hexanes then 100% EtOAc) to afford **54** as a viscous colorless oil (0.5 g, 29%).

**Step 5. Preparation of lithium 10-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(2-hydroxyethyl)amino)-10-oxodecanoate 55**



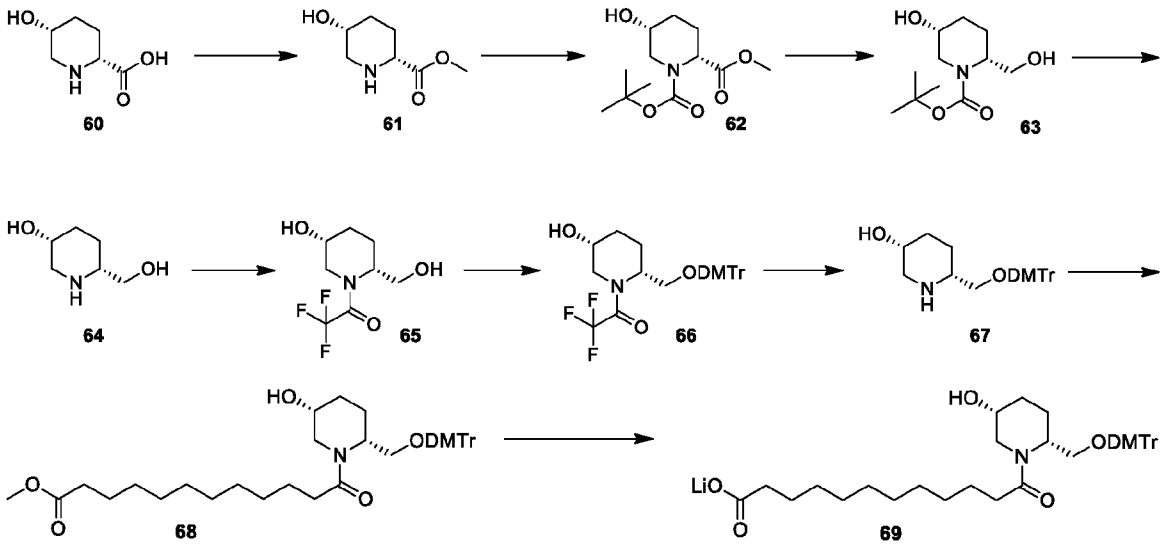
To a solution of methyl 10-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(2-hydroxyethyl)amino)-10-oxodecanoate **54** (0.5 g, 0.83 mmol) in THF (40 mL) was added water (10 mL) and lithium hydroxide (24 mg, 1.0 mmol). The solution was stirred overnight at room temperature then concentrated in vacuo to remove the THF. The remaining aqueous solution was flash frozen on liquid nitrogen and lyophilized to afford **55** as a colorless solid (485 mg, 95%).

**Step 6. Preparation of compounds 56, 57, 58 and 50**

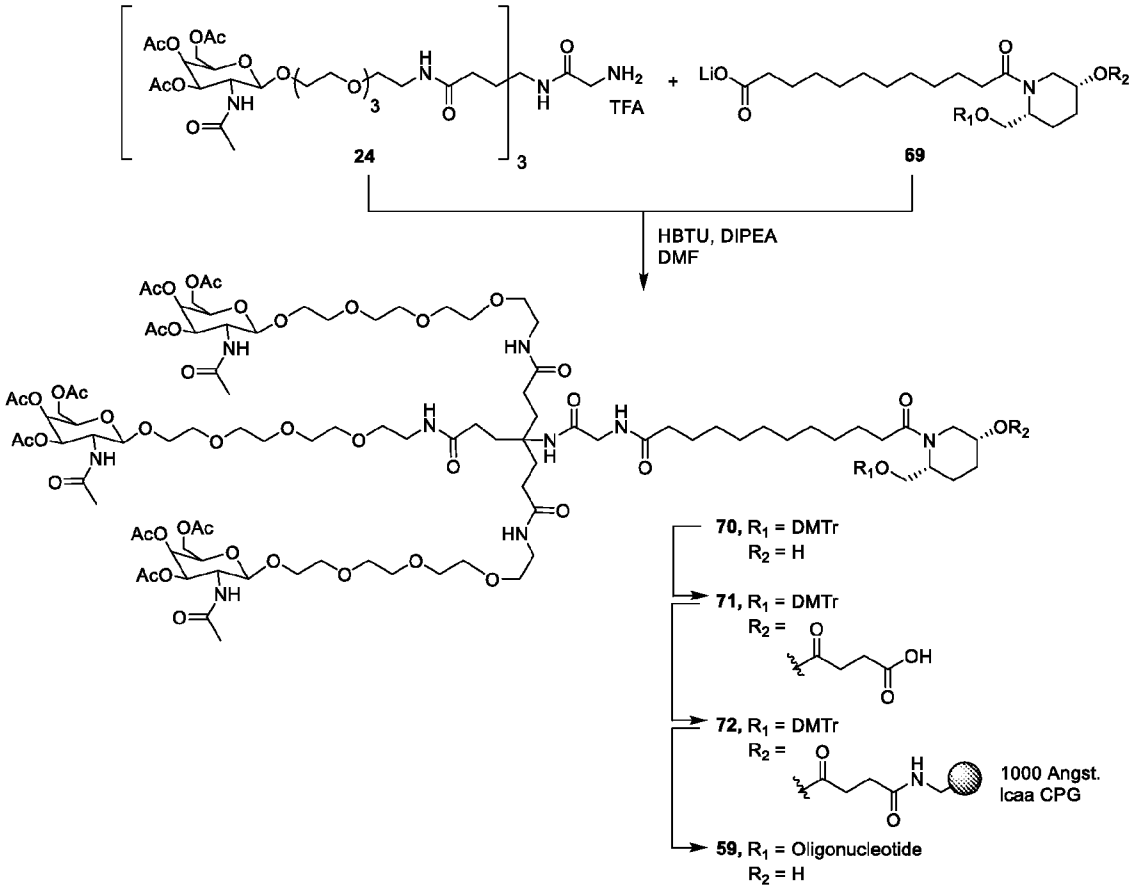
Compounds **56**, **57**, **58** and **50** were prepared using the identical procedures to those used to synthesize compounds **47**, **48**, **49** and **43** respectfully.

**Example 7. Synthesis of conjugate 59**

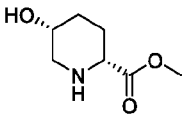
**Scheme 15.**



**Scheme 16.**

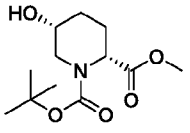



**Step 1. Preparation of methyl (2R,5R)-5-hydroxypiperidine-2-carboxylate 61**



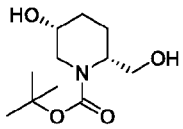
(2R,5R)-5-hydroxypiperidine-2-carboxylic acid **60** (3.5 g, 24.1 mmol) was stirred in MeOH (50 mL). HCl (g) was bubbled through the solution for 2 mins and the reaction stirred at reflux for 1.5 h. The reaction was concentrated in-vacuo to give methyl (2R,5R)-5-hydroxypiperidine-2-carboxylate **61** in quantitative yield which was used without further purification.

**Step 2. Preparation of 1-(tert-butyl) 2-methyl (2R,5R)-5-hydroxypiperidine-1,2-dicarboxylate 62**



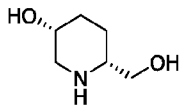
Methyl (2R,5R)-5-hydroxypiperidine-2-carboxylate **61** (24.1 mmol) and TEA (7.2 mL, 53.02 mmol) were stirred in DCM (100 mL) at RT. Di-*tert*-butyl-di-carbonate (5.7 g, 26.5 mmol) was added in portions and the reaction stirred for 2 h. The reaction was diluted with DCM (100 mL) and washed sequentially with 1 M HCl (2 x 75 mL), saturated $NaHCO_3$ (2 x 75 mL), $H_2O$ (2 x 75 mL) and saturated NaCl solution (2 x75 mL). The organics were separated, dried ($Na_2SO_4$) and concentrated in-vacuo to give 1-(tert-butyl) 2-methyl (2R,5R)-5-hydroxypiperidine-1,2-dicarboxylate **62** (5.53 g, 88%) which was used without further purification.

**Step 3. Preparation of tert-butyl (2R,5R)-5-hydroxy-2-(hydroxymethyl)piperidine-1-carboxylate 63**



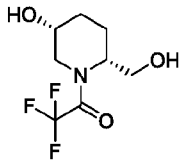

(2R,5R)-1-(tert-Butoxycarbonyl)-5-hydroxypiperidine-2-carboxylic acid **62** (5.53 g, 21.4 mmol) was stirred in THF at 0°C. $LiBH_4$ (3.0 M solution in THF)(8.9 mL, 27.7 mmol) was added dropwise over 1 hr. The reaction was allowed to warm to RT and stirring continued for 16 h. Reaction was quenched with 1M NaOH, THF removed in-vacuo and the aqueous exhaustively extracted with EtOAc (10 x 100 mL). The combined organics were washed with $H_2O$ (50 mL), saturated NaCl solution (2 x 50 mL), dried ($Na_2SO_4$) and concentrated in-vacuo to give *tert*-butyl (2R,5R)-5-hydroxy-2-(hydroxymethyl)piperidine-1-carboxylate **63** (2.4 g, 49.0 %) which was used without further purification.

**Step 4. Preparation of (3R,6R)-6-(hydroxymethyl)piperidin-3-ol 64**



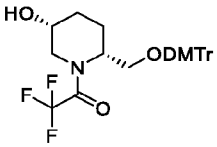

*tert*-Butyl (2R,5R)-5-hydroxy-2-(hydroxymethyl)piperidine-1-carboxylate **63** (2.4 g, 10.4 mmol) was stirred in $Et_2O$ at RT. HCl (g) was bubbled through for 45 secs and the reaction stirred at RT for 45 mins. The reaction was concentrated in-vacuo and dried under hi-vac to afford (3R,6R)-6-(hydroxymethyl)piperidin-3-ol **64**. The product was used without further purification.

**Step 5. Preparation of 2,2,2-trifluoro-1-((2R,5R)-5-hydroxy-2-(hydroxymethyl)piperidin-1-yl)ethan-1-one 65**



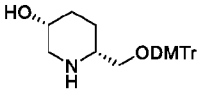
Crude (3R,6R)-6-(hydroxymethyl)piperidin-3-ol **64** from the previous reaction was stirred in MeCN (50 mL) with TEA (3.5 mL, 25.2 mmol) at RT. Ethyl trifluoroacetate (3 mL, 25.2 mmol) was added and the reaction stirred at RT for 16 hr, then concentrated in-vacuo to give 2,2,2-trifluoro-1-((2R,5R)-5-hydroxy-2-(hydroxymethyl)piperidin-1-yl)ethan-1-one **65**. The product was used without further purification.

**Step 6. Preparation of 1-((2R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-hydroxypiperidin-1-yl)-2,2,2-trifluoroethan-1-one 66**



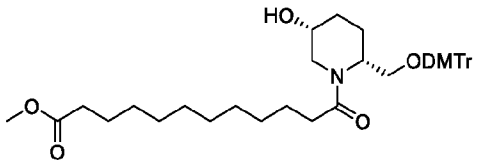
Crude 2,2,2-trifluoro-1-((2R,5R)-5-hydroxy-2-(hydroxymethyl)piperidin-1-yl)ethan-1-one **65** from the previous reaction was stirred in DCM with TEA (50 mL) at RT. 4,4'-Dimethoxytrityl chloride (DMTrCl) (3.87 g, 11.44 mmol) was added in one portion and the reaction stirred at RT for 3 hours. The reaction was diluted with DCM (50 mL) and washed sequentially with saturated $NaHCO_3$ (2 x 75 mL), $H_2O$ (2 x 75 mL) and saturated NaCl solution (2 x75 mL). The organics were separated, dried ($Na_2SO_4$), concentrated in-vacuo and purified by column chromatography (100% hexanes – 60% EtOAc/Hexanes) (0.1 % TEA) to give 1-((2R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-hydroxypiperidin-1-yl)-2,2,2-trifluoroethan-1-one **66** (3.14 g, 57%)

**Step 7. Preparation of (3R,6R)-6-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-piperidin-3-ol 67**



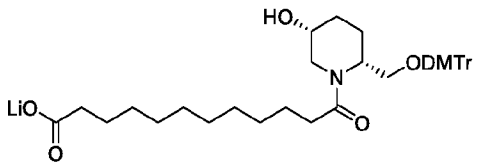
1-((2R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-hydroxypiperidin-1-yl)-2,2,2-trifluoroethan-1-one **66** (3.14 g, 6.0 mmol) was stirred in MeOH (50 mL) at RT. KOH (672 mg, 12 mmol) was added and the reaction stirred at RT for 16 hours. Additional KOH (300 mg, 6 mmol) was added and stirring continued for an additional 24 h. The reaction was concentrated in-vacuo, taken up in DCM (150 mL), washed with $H_2O$ (4 x 50 mL), dried ($Na_2SO_4$) and concentrated in-vacuo to give (3R,6R)-6-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)piperidin-3-ol **67** (2.34 g, 90%) which was used without further purification.

**Step 8. Preparation of methyl 12-((2R,5R)-2-((bis(4-methoxyphenyl)(phenyl)-methoxy)methyl)-5-hydroxypiperidin-1-yl)-12-oxododecanoate 68**




(3R,6R)-6-((Bis(4-methoxyphenyl)(phenyl)methoxy)methyl)piperidin-3-ol **67** (2.34 g, 5.34 mmol) was stirred in DCM (75 mL) at RT. Triethylamine (2.2 mL, 16.2 mmol), HATU (3.5 g, 9.2 mmol) and 12-methoxy-12-oxododecanoic acid (1.32 g, 5.4 mmol) were added and the reaction stirred at RT for 3 h. The resultant solid precipitate was removed by filtration, the filtrate concentrated in-vacuo and the residue purified by column chromatography (2.5 %MeOH/DCM, 0.1% TEA) to give methyl 12-((2R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-hydroxypiperidin-1-yl)-12-oxododecanoate **68** in quantitative yield.

**Step 9. Preparation of lithium 12-((2R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)-methyl)-5-hydroxypiperidin-1-yl)-12-oxododecanoate 69**




Methyl 12-((2R,5R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-5-hydroxypiperidin-1-yl)-12-oxododecanoate **68** (5.4 mmol) and LiOH (140 mg, 5.94 mmol) were stirred in THF:$H_2O$ (1:1, 100 mL) at RT for 48 h. The THF was removed in-vacuo, the aqueous frozen and lyophilized to give lithium 12-((2R,5R)-2-((bis(4-

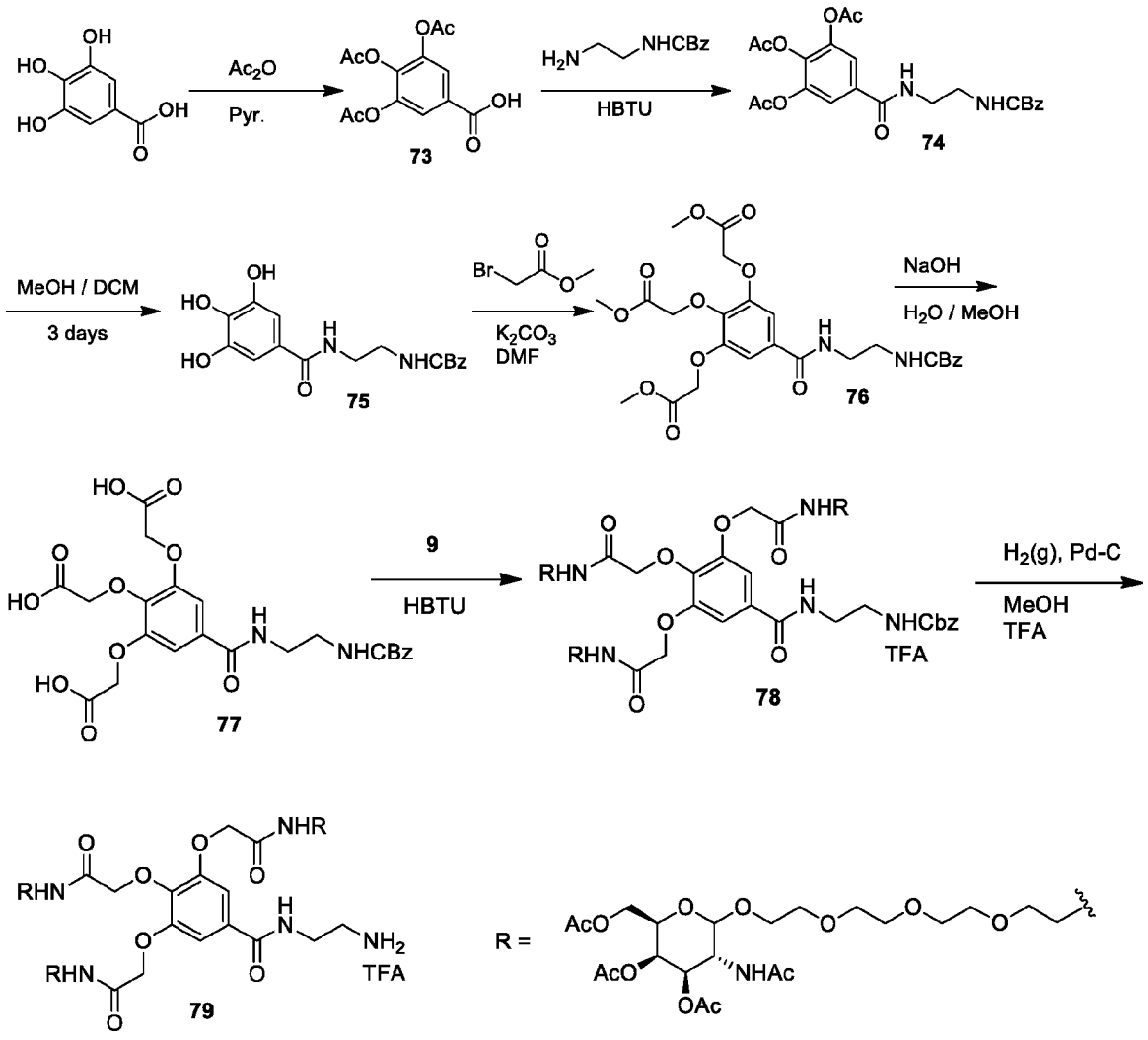
methoxyphenyl)(phenyl)methoxy)methyl)-5-hydroxypiperidin-1-yl)-12-oxododecanoate **69** (3.2 g, 91 %). Which was used in subsequent reactions without additional purification.

**Step 10. Preparation of compounds 70, 71, 72, and 59**

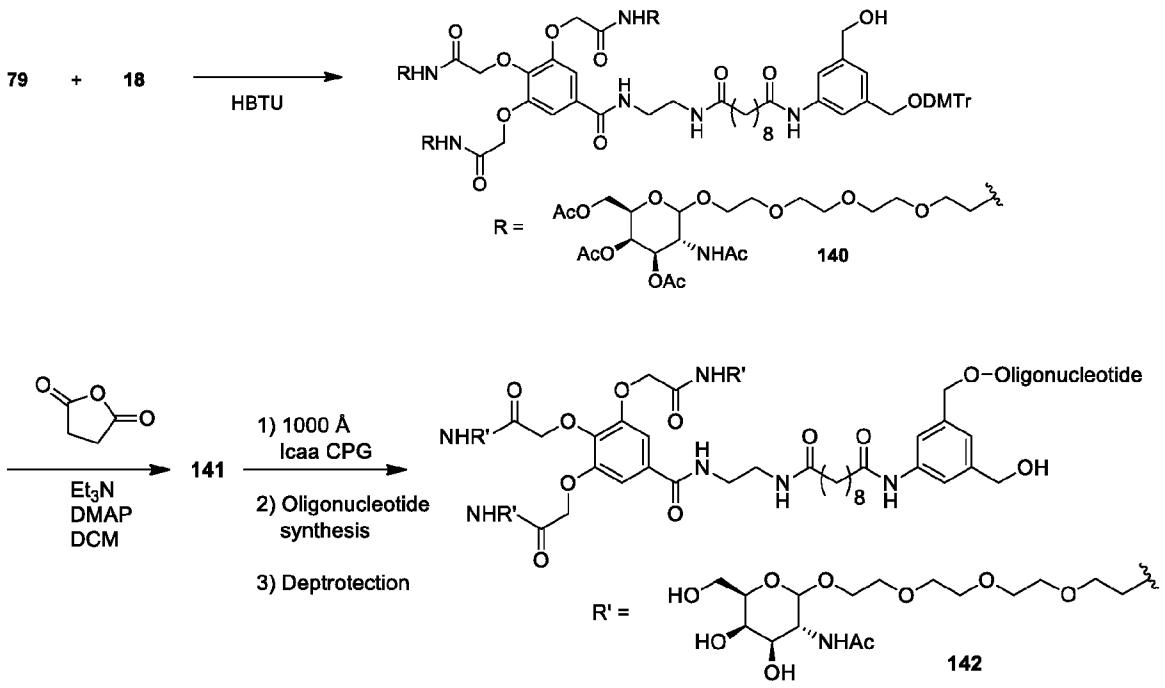
Compounds **70**, **71**, **72** and **59** were prepared using the identical procedures to those used to synthesize compounds **47**, **48**, **49** and **43** respectfully.

**Example 8. Synthesis of conjugate 142**

**Scheme 17.**



**Scheme 18.**




**Step 1. Preparation of 3,4,5-Triacetoxybenzoic acid 73**

To a solution of Gallic acid (20 g) in pyridine (50 mL) and acetic anhydride (50 mL). The solution was stirred overnight at room temperature then poured into ice water (1 L). The solution was made acidic with concentrated hydrochloric acid where upon a colorless solid precipitated. The solid was collected via filtration and washed with water (5 x 100 mL). The wet solid was frozen on liquid nitrogen and freeze dried to afford 3,4,5-triacetoxybenzoic acid (26 g, 75%).

**Step 2. Preparation of 5-((2-((2-Oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)carbamoyl)benzene-1,2,3-triyl triacetate 74**

To a solution of 3,4,5-triacetoxybenzoic acid (10 g, 33.8 mmol), N-carbobenzoxy-1,2-diaminoethane hydrochloride (5.3 g, 33.8 mmol) and HBTU (13.5 g, 35.5 mmol) in DMF (200 mL) was added DIPEA (17.5 mL, 101 mmol). The solution was stirred for 16 hours then diluted with ethyl acetate (250 mL), washed with brine (3 x 200 mL), dried on magnesium sulfate, filtered and concentrated *in vacuo* to dryness. The crude product was purified by column chromatography on silica gel (Gradient 1% to 5% MeOH in DCM) to afford 5-((2-((2-Oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)carbamoyl)benzene-1,2,3-triyl triacetate as an off white solid (5.5 g).

**Step 3. Preparation of 3,4,5-Trihydroxy-N-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)benzamide 75**

A solution of 5-((2-((2-Oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)carbamoyl)benzene-1,2,3-triyl triacetate (5 g, 1.1 mmol) in 1:1 MeOH / $CH_2Cl_2$ (100 mL) was stirred for 3 days at room temperature. Upon completion the solvent was removed to afford 3,4,5-Trihydroxy-N-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)benzamide as a colorless solid (4 g, quantitative).

**Step 4. Preparation of Trimethyl 2,2',2''-((5-((2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)carbamoyl)benzene-1,2,3-triyl)tris(oxy))triacetate 76**

A solution of 3,4,5-Trihydroxy-N-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)benzamide (4 g, 11.6 mmol), methyl bromoacetate (7.7 g, 46.4 mmol) and potassium carbonate (9.6 g, 69.4 mmol) in DMF (100 mL) was stirred overnight at 60 °C. Upon completion the solution was cooled to room temperature, diluted with ethyl acetate (200 mL), washed with water (200 mL), brine (3 x 100 mL), dried on magnesium sulfate, filtered and concentrated in vacuo to dryness. The crude product was purified by column chromatography on silica gel (Gradient 2% to 10% MeOH in DCM) to afford trimethyl 2,2',2''-((5-((2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)carbamoyl)benzene-1,2,3-triyl)tris(oxy))-triacetate as a beige solid (5 g, 79%)

**Step 5. Preparation of 2,2',2''-((5-((2-((2-Oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)-carbamoyl)benzene-1,2,3-triyl)tris(oxy))triacetic acid 77**

A solution of trimethyl 2,2',2''-((5-((2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)-carbamoyl)benzene-1,2,3-triyl)tris(oxy))triacetate (5 g, 9.2 mmol) and 1M NaOH (30 mL) in methanol (100 mL) was stirred for 2 hours at room temperature. Upon completion the reaction was concentrated to remove the methanol and diluted with water (75 mL). The mixture was cooled to 0°C, acidified with 2M HCl and extracted with ethyl acetate (5 x 150 mL). The combined ethyl acetate extracts were dried on magnesium sulfate, filtered and concentrated in vacuo to dryness to afford 2,2',2''-((5-((2-((2-Oxo-2-phenyl-1λ$^2$-ethyl)amino)ethyl)carbamoyl)benzene-1,2,3-triyl)tris(oxy))triacetic acid as a colorless solid (2.3 g, 50%).

**Step 6. Preparation of Compound 78**

Compound **78** was prepared from compounds **9** (2.75 g, 4.3 mmol) and **77** (0.5 g, 0.96 mmol) using an identical procedure to that used for compound **13**. Yield: 600 mg.

**Step 7. Preparation of Compound 79**

Compound **79** was prepared from compounds **78** (0.6 g) using an identical procedure to that used for compound **14**. Yield: 500 mg.

**Step 8. Preparation of compound 140**

Compound **140** was prepared from compound **79** (500 mg, 0.25 mmol) and compound **18** (175 mg, 0.25 mmol) using an identical procedure to that used for compound **19**. Yield: 250 mg, 44%.

**Step 9. Preparation of compound 141**

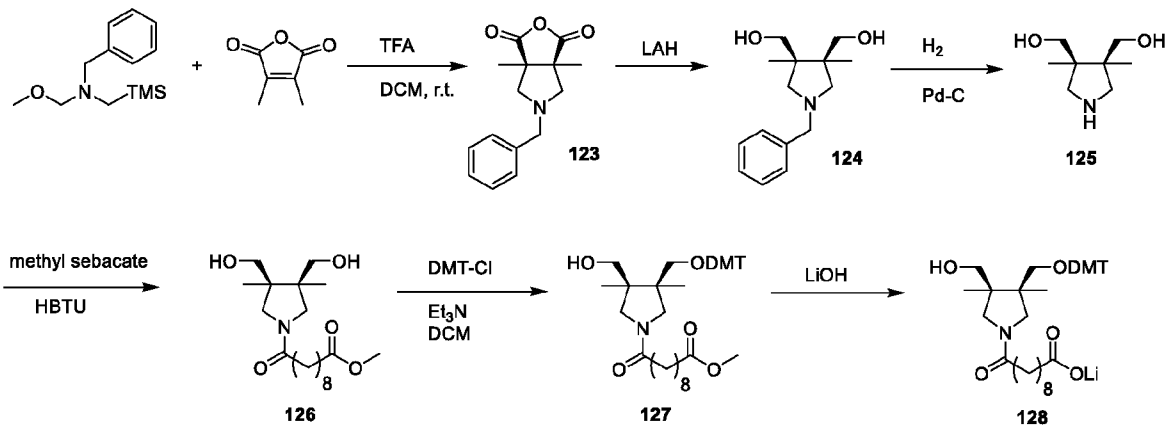
Compound **141** was prepared from compound **140** (250 mg, 0.11 mmol) using an identical procedure to that used for compound **20**. Yield: 200 mg

**Step 10. Preparation of conjugate 142**

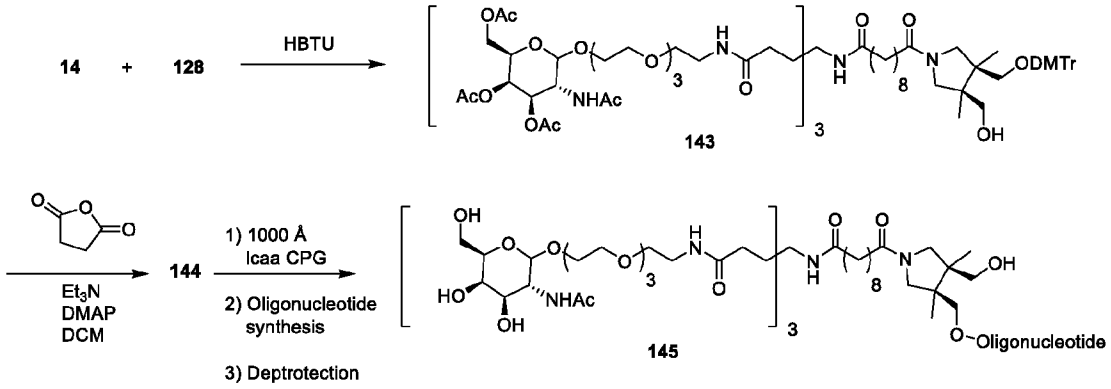
Conjugate **142** was prepared from compound **141** (200 mg) and 1000A lcaa CPG (1.8 g) using an identical procedure to that used for compound **1**. Yield: 1.9 g, 22 μmol/g CPG loading. The resulting GalNAc loaded CPG solid support was employed in automated oligonucleotide synthesis using standard procedures. Nucleotide deprotection followed by removal from the solid support (with concurrent galactosamine acetate deprotection) afforded the GalNAc-oligonucleotide conjugate **142.**

**Example 9. Synthesis of conjugate 145**

**Scheme 19.**



**Scheme 20.**




**Step 1. Preparation of (3aR,6aS)-5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione 123**

To a cooled solution (0°C) of 3,4-dimethylfuran-2,5-dione (3 g, 24 mmol) and N-benzyl-1-methoxy-N-((trimethylsilyl)methyl)methanamine (7 g, 29.8 mmol) in dichloromethane (75 mL) was slowly added trifluoroacetic acid (75 µL). Stir overnight allowing the solution to slowly warm to room temperature as the ice bath melted. The reaction mixture was concentrated to dryness, dissolved in ethyl acetate (100 mL), washed with saturated sodium bicarbonate (2 x 100mL), dried on magnesium sulfate, filtered and concentrated to dryness. Purification by column chromatography on silica gel (gradient: 20% ethyl acetate in hexanes to 100% ethyl acetate) afforded (3aR,6aS)-5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione as a yellow oil (3.5 g, 56%)

**Step 2. Preparation of ((3R,4S)-1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol 124**

To a cooled (0°C) solution of (3aR,6aS)-5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione (3.5 g, 13.4 mmol) in anhydrous diethyl ether (50 mL) was added slowly lithium aluminum hydride pellets (1.5 g, 40 mmol) over three portions. The solution was stirred overnight warming to room temperature as the ice water bath melted. Upon completion, the reaction was cooled to 0°C and very slowly quenched with 1.5 mL of 5M NaOH followed by 1.5 mL of water. Stir for 30 minutes then add magnesium sulfate and filter. The filtrate was concentrated to afford ((3R,4S)-1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol as a colorless oil (2.7 g)

**Step 3. Preparation of ((3R,4S)-3,4-Dimethylpyrrolidine-3,4-diyl)dimethanol 125**

To a solution of ((3R,4S)-1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol (10 g, 40 mmol) in methanol (10 mL) was added 10% palladium on activated charcoal wet (1 g). The solution was stirred vigorously under a hydrogen atmosphere for 16 hours. Upon completion the solution was filtered through Celite, and concentrated to dryness to afford ((3R,4S)-3,4-Dimethylpyrrolidine-3,4-diyl)dimethanol as a colorless solid (5.5 g, 86%).

**Step 4. Preparation of Methyl 10-((3R,4S)-3,4-bis(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 126**

Compound **126** was prepared from compound **125** (1.3 g, 8.2 mmol) and monomethyl sebacate (1.8 g, 8.2 mmol) using an identical procedure to that used for compound **17**. Yield: 1.8 g, 61%.

**Step 5. Preparation of Methyl 10-((3R,4S)-3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 127**

Compound **127** was prepared from compound **126** (1.8 g, 5.0 mmol) and 4,4′-Dimethoxytrityl chloride (1.7 g, 5.0 mmol) using an identical procedure to that used for compound **18**. Yield: 1.4 g, 42%.

**Step 6. Preparation of Lithium 10-((3R,4S)-3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 128**

To a solution of compound **127** (3.0 g, 4.6 mmol) in THF (50 mL) and water (50 mL) was added lithium hydroxide (121 mg, 5.0 mmol). The solution was stirred for 4 hours at room temperature then concentrated to remove the THF. The remaining aqueous solution was freeze dried overnight to afford a pale pink solid (2.9 g, quantitative)

**Step 7. Preparation of compound 143**

Compound **143** was prepared from compound **128** (270 mg, 0.42 mmol) and compound **14** (800 mg, 0.42 mmol) using an identical procedure to that used for compound **19**. Yield: 900 mg, 87%.

**Step 8. Preparation of compound 144**

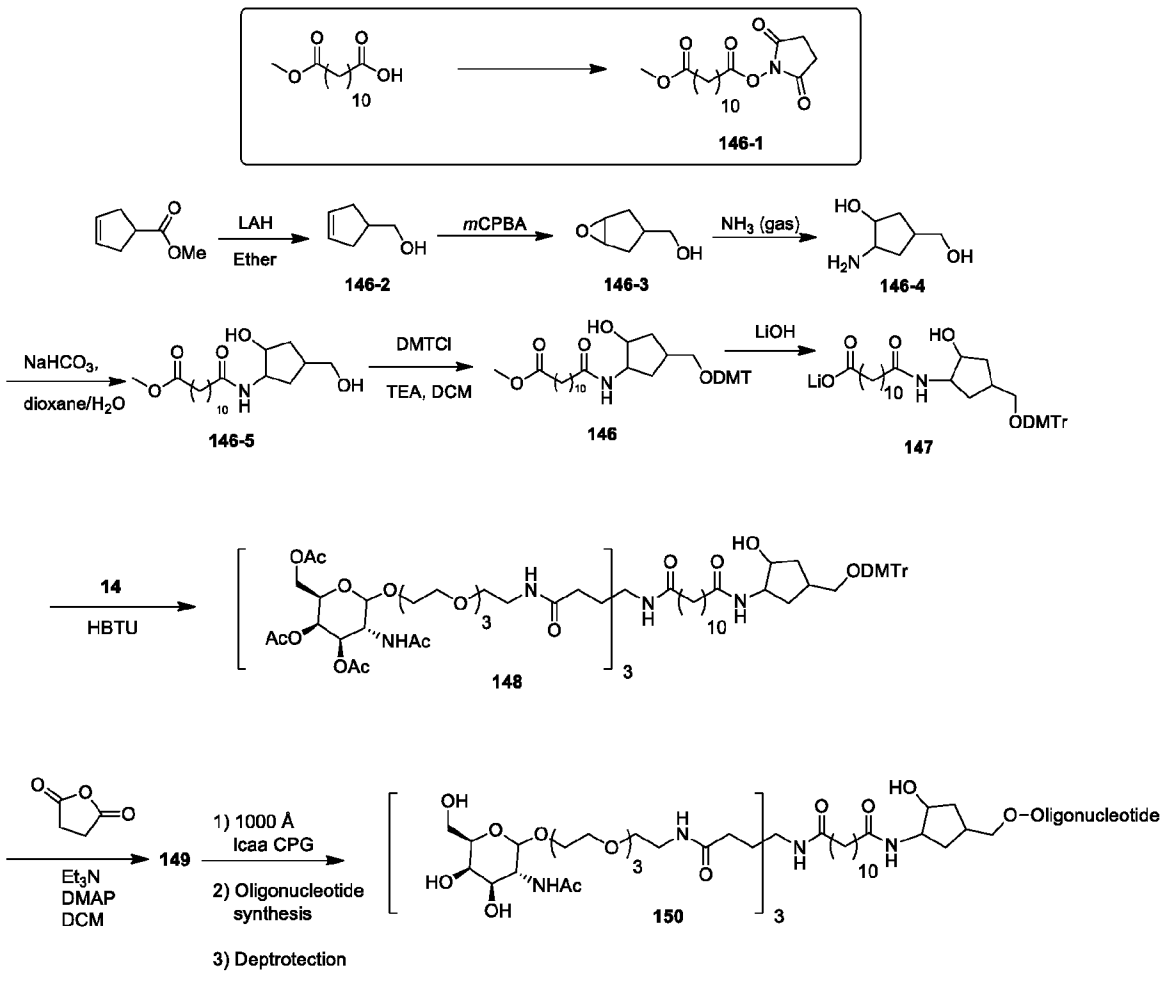
Compound **144** was prepared from compound **143** (500 mg, 0.2 mmol) using an identical procedure to that used for compound **20**. Yield: 200 mg

**Step 9. Preparation of conjugate 145**

Conjugate **145** was prepared from compound **144** (200 mg) and 1000A lcaa CPG (1.8 g) using an identical procedure to that used for compound **1**. Yield: 1.9 g, 20 μmol/g CPG loading. The resulting GalNAc loaded CPG solid support was employed in automated oligonucleotide synthesis using standard procedures. Nucleotide deprotection followed by removal from the solid support (with concurrent galactosamine acetate deprotection) afforded the GalNAc-oligonucleotide conjugate **145.**

**Example 10. Synthesis of conjugate 150**

**Scheme 21.**



**Step 1. Preparation of 146-1**

To a solution of mono methyl ester of dodecanedioic acid (12.2 g, 50.0 mmol) in dichloromethane (300 mL) was added N-hydroxysuccinimide (6.10g, 53.0 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (10.52g, 55.0 mmol). The cloudy mixture was stirred overnight at room temperature and the reaction became a clear solution. TLC indicated the reaction was completed. The organics were washed with saturated $NH_4Cl$ (300 mL) and brine (100 mL). The organic layer was separated, dried over $MgSO_4$ and concentrated to dryness to pure 1-(2,5-dioxopyrrolidin-1-yl) 12-methyl dodecanedioate **146-1** as a white solid (16.7g, 97.8%).

**Step 2. Preparation of cyclopent-3-en-1-ylmethanol 146-2**

To a suspension of lithium aluminum hydride (15.2g, 0.40 mol) in anhydrous ether (1 L) at 0°C under nitrogen, was added the solution of methyl cyclopent-3-enecarboxylate (50 g, 0.40 mol) in ether (300 mL) dropwise over 5 hrs. The suspension was stirred at room temperature overnight. TLC indicated the completion of the reaction. The reaction was re-cooled to 0°C. Saturated solution of $Na_2SO_4$ (32 mL) was added dropwise to quench the reaction. After the addition was complete, the mixture was stirred for another 3 hrs and was filtered through a pad of celite. Evaporation of solvent afforded cyclopent-3-enylmethanol **146-2** (37.3 g, 95 %) as a colorless liquid.

**Step 3. Preparation of (6-oxabicyclo[3.1.0]hexan-3-yl)methanol 146-3**

To a solution of cyclopent-3-enylmethanol **146-2** (4.0 g, 41 mmol) in dichloromethane (150 mL) at 0°C was added 3-chloroperbenzoic acid (10 g, 45 mmol, 77% purity) by portion. The reaction was stirred overnight. Dichloromethane (150 mL) was added. The organics was washed with sodium thiosulfate (12 g in 10 mL water), followed by saturated $NaHCO_3$ (40 mL). This was repeated till all the remaining 3-chloroperbenzoic acid was washed away. The organic was dried over $MgSO_4$. Evaporation of solvent gave a mixture of *cis*- and *trans*- 6-oxabicyclo[3.1.0]hexan-3-ylmethanol **146-3** (2.6 g, 57 %) as a yellow oil. GC-MS: *m/z* 114 (5) ($M^+$), 95 (15), 88 (100), 81 (15).

**Step 4. Preparation of 2-amino-4-(hydroxymethyl)cyclopentan-1-ol 146-4**

To a solution of 6-oxabicyclo[3.1.0]hexan-3-ylmethanol **146-3** (2.0g, 17.6 mmol) in methanol (20 mL) at 0°C was purged ammonia gas for 10 min. The reaction was stirred at room temperature overnight. TLC indicated the incompletion of the reaction. Methanol was removed

and $NH_3 \cdot H_2O$ (50 mL) was added and this was stirred at room temperature over a week. TLC confirmed the completion of the reaction. Water was removed by azeotropically with ethanol to afford 2-amino-4-(hydroxymethyl)cyclopentanol **146-4** (2.1 g, 91%) as a yellow oil.

**Step 5. Preparation of Methyl 12-(2-hydroxy-4-(hydroxymethyl)cyclopentylamino)-12-oxododecanoate 146-5**

Compound **146-5** was prepared from 2-amino-4-(hydroxymethyl)cyclopentanol **146-4** and 1-(2,5-dioxopyrrolidin-1-yl) 12-methyl dodecanedioate **146-1**, using the same procedure as described in the synthesis of 12-(2-(*tert*-butoxycarbonylamino)ethylamino)-12-oxododecanoate (3-2). Methyl 12-(2-hydroxy-4-(hydroxymethyl)cyclopentylamino)-12-oxododecanoate **146-5** was obtained in 87.4% yield as an off-white solid.

**Step 6. Preparation of compound 147**

Compound **147** was prepared quantitatively from compound **146** (1.4 g, 2.33mmol) using an identical procedure to that used for compound **18**.

**Step 7. Preparation of compound 148**

Compound **148** was prepared from compound **147** (150mg, 0.23mmol) and compound **14** (431mg, 0.23mmol) using an identical procedure to that used for compound **19**. Yield: 460mg, 84%.

**Step 8. Preparation of compound 149**

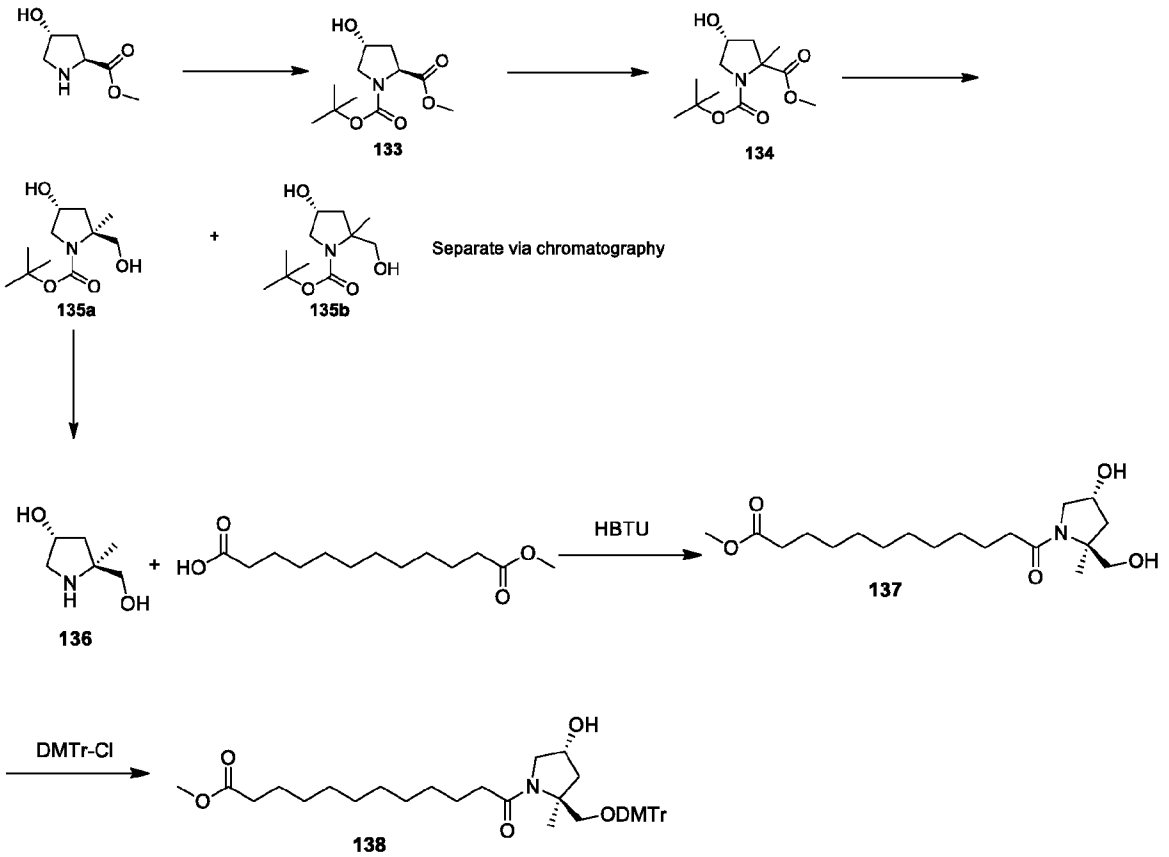
Compound **149** was prepared from compound **148** (460mg, 0.19mmol) using an identical procedure to that used for compound **20**. Yield: 436mg, 91%.

**Step 9. Preparation of conjugate 150**

Compound **150** was prepared from compound **149** (436mg) and 1000A lcaa CPG (2.62g) using an identical procedure to that used for compound **1**. Yield: 2.7g, 21.3μmol/g CPG loading. The resulting GalNAc loaded CPG solid support was employed in automated oligonucleotide synthesis using standard procedures. Nucleotide deprotection followed by removal from the solid support (with concurrent galactosamine acetate deprotection) afforded the GalNAc-oligonucleotide conjugate **150.**

**Example 11. Synthesis of conjugates 153, 158, 163, 168 and 173**

**Scheme 22.**




**Step 1. Preparation of 1-(tert-butyl) 2-methyl (2S,4R)-4-hydroxypyrrolidine-1,2-dicarboxylate (133)**

Methyl (2S,4R)-4-hydroxypyrrolidine-2-carboxylate (25.9 g, 46 mmol), BOC anhydride (65.9 g, 302.5 mmol) and TEA (42 ml, 302.5 mmol) were stirred in DCM at RT for 16 h. The organics were washed sequentially with 1M HCl (x2), saturated $NaHCO_3$ (x2), $H_2O$ and brine, dried and concentrated in-vacuo to give 1-(tert-butyl) 2-methyl (2S,4R)-4-hydroxypyrrolidine-1,2-dicarboxylate (**133**) (58.1g, 85%)

**Step 2. Preparation of 1-(tert-butyl) 2-methyl (4R)-4-hydroxy-2-methylpyrrolidine-1,2-dicarboxylate (134)**

1-(tert-butyl) 2-methyl (2S,4R)-4-hydroxypyrrolidine-1,2-dicarboxylate (**133**) (5g, 20.4 mmol) and MeI (12 g, 84.5 mmol) were stirred in anhydrous THF at -40°C. LDA (2.0 M solution in THF) (37.5 mL, 75 mmol) was added dropwise. The reaction was allowed to warm to RT and stirred for 4 h then quenched with saturated $NH_4Cl$. The reaction was extracted with EtOAc, washed with $H_2O$ and brine, dried ($Na_2SO_4$) and concentrated in-vacuo. The residue was

purified by column chromatography 50:50 EtOAc//hexanes to give 1-(tert-butyl) 2-methyl (4R)-4-hydroxy-2-methylpyrrolidine-1,2-dicarboxylate **(134)** as a racemic mixture (3.6 g, 68%)

**Step 3. Preparation of tert-butyl (2S,4R)-4-hydroxy-2-(hydroxymethyl)-2-methylpyrrolidine-1-carboxylate (135a)**

1-(Tert-butyl) 2-methyl (4R)-4-hydroxy-2-methylpyrrolidine-1,2-dicarboxylate **(134)** (19g, 73.5 mmol) was stirred in anhydrous THF under $N_2$. $LiBH_4$ solution (48 ml, 96 mmol) was added dropwise and the reaction stirred at RT for 48 h. The reaction was quenched with 1M NaOH, the THF removed in-vacuo and the residual extracted with EtOAc (4 x 100ml). The organics were washed with $H_2O$ and brine, dried ($Na_2SO_4$) and concentrated in-vacuo. The residue was purified by column chromatography (5% MeOH/DCM) to give tert-butyl (2S,4R)-4-hydroxy-2-(hydroxymethyl)-2-methylpyrrolidine-1-carboxylate (**135a**) as the major product (8g, 47%). Structure assigned according to literature references

**Step 4. Preparation of (3R,5S)-5-(hydroxymethyl)-5-methylpyrrolidin-3-ol hydrochloride (136)**

tert-Butyl (2S,4R)-4-hydroxy-2-(hydroxymethyl)-2-methylpyrrolidine-1-carboxylate (**135a**) (8g, 34.6 mmol) was stirred in EtOAc at RT and gaseous HCl applied for approximately two minutes. The reaction was stirred for one hour then concentrated in-vacuo and dried under high vacuum to give (3R,5S)-5-(hydroxymethyl)-5-methylpyrrolidin-3-ol hydrochloride **(136)** in quantitative fashion.

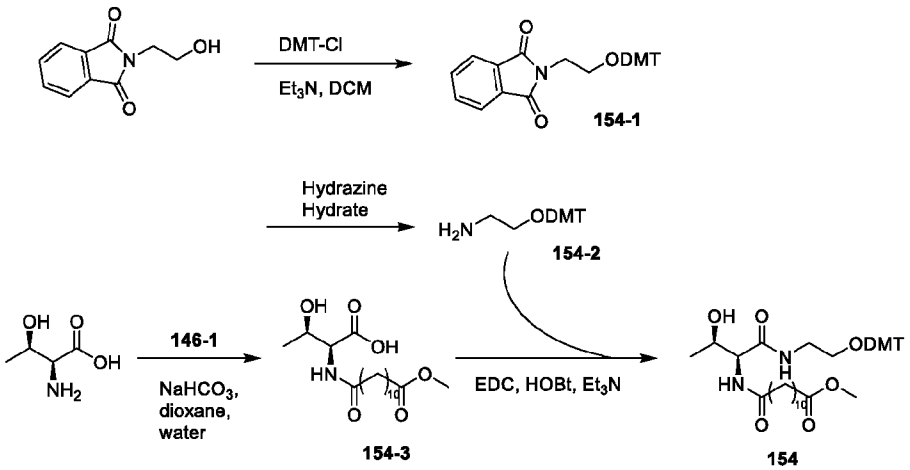
**Step 5. Preparation of methyl 12-((2S,4R)-4-hydroxy-2-(hydroxymethyl)-2-methylpyrrolidin-1-yl)-12-oxododecanoate (137)**

(3R,5S)-5-(Hydroxymethyl)-5-methylpyrrolidin-3-ol hydrochloride **(136)** (7.9 g, 47.4 mmol), 12-methoxy-12-oxododecanoic acid (11.5 g, 47.4 mmol), HBTU (36 g, 76 mmol) and TEA 20 mL, 142.2 mmol) were stirred in DCM at RT for 16h. The precipitate was removed by filtration and the organics washed with 1M HCl (x2), saturated $NaHCO_3$ (x2), $H_2O$ and brine. After drying the organics were concentrated in-vacuo and purified by column chromatography (5%MeOH/DCM) to give methyl 12-((2S,4R)-4-hydroxy-2-(hydroxymethyl)-2-methylpyrrolidin-1-yl)-12-oxododecanoate (**137**) (3.1 g, 18.3 %)

**Step 6. Preparation of methyl 12-((2S,4R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxy-2-methylpyrrolidin-1-yl)-12-oxododecanoate (138)**

Methyl 12-((2S,4R)-4-hydroxy-2-(hydroxymethyl)-2-methylpyrrolidin-1-yl)-12-oxododecanoate (**137**) (3.1 g, 9.0 mmol), DMTr-Cl (2.8 g, 8.2 mmol) and TEA (1.1 ml, 8.2 mmol) were stirred in DC< at RT for 16 h. The reaction was concentrated in-vacuo and the residue purified by column chromatography (5% MeOH/DCM, 0.1%TEA) to give methyl 12-((2S,4R)-2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-hydroxy-2-methylpyrrolidin-1-yl)-12-oxododecanoate (**138**) (2.7 g, 45.5 mmol).

**Scheme 23**




**Step 7. Preparation of Compound 154-1**

To a solution of *N*-(2-hydroxyethyl)phthalimide (4.80 g, 25.0 mmol) and 4,4'-dimethoxytrityl chloride (8.8 g, 26.0 mmol) in dichloromethane (200 mL) at 0°C under nitrogen, was added triethylamine (10.4 mL, 74.6 mmol) dropwise. The reaction mixture was stirred at room temperature for 3 hrs. TLC indicated the completion of the reaction. The organic layer was washed with brine (100 mL), dried over $MgSO_4$, and concentrated to dryness. This was used directly for the next reaction without purification.

**Step 8. Preparation of Compound 154-2**

2-(2-(Bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)isoindoline-1,3-dione (**154-1**) obtained above and hydrazine monohydrate (3.6 mL, 74 mmol) in ethanol (100 mL) was stirred overnight at room temperature. TLC indicated the completion of the reaction. The precipitate was filtered out. The filtrate was evaporated. The residue was taken up by ethyl acetate (100

mL). The organic solution was washed with 10% NaOH, water and brine, and dried over $MgSO_4$. Evaporation of solvent afforded 2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethanamine (**154-2**) as a yellow liquid (8.11g, 89.3% yield over two steps). This was used for the next reaction without further purification.

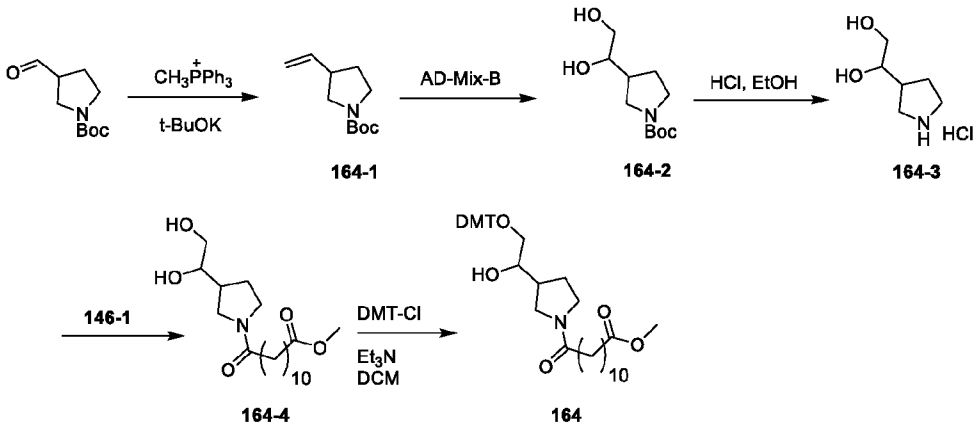
**Step 9. Preparation of Compound 154-3**

To a solution of L-threonine (1.19g, 10.0 mmol) and $NaHCO_3$ (2.3g, 27 mmol) in water (20 mL) and dioxane (10 mL), was added 1-(2,5-dioxopyrrolidin-1-yl) 12-methyl dodecanedioate **146-1** (3.1g, 9.1 mmol) in dioxane (10 mL) dropwise. The reaction mixture was stirred at room temperature overnight. 4N HCl (10 mL) was added. The precipitate was collected by filtration and washed with water (3 x 10 mL). The solid was dried over $P_2O_5$ in a desiccator to afford (2S,3R)-3-hydroxy-2-(12-methoxy-12-oxododecanamido)butanoic acid **154-3** as an off-white solid (2.84g, 82.2%). LC-MS (ESI): *m/z*: 346 (100), $(M + H^+)$.

**Step 10. Preparation of Compound 154**

(2S,3R)-3-hydroxy-2-(12-methoxy-12-oxododecanamido)butanoic acid **154-3** (2.47g, 7.15 mmol), 2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethanamine **154-2** (2.60g, 7.15 mmol), EDC (1.64g, 8.58 mmol), 1-hydroxybenzotriazole (HOBt) (1.16g, 8.58 mmol) and TEA (2.4 mL, 17.2 mmol) were stirred in dichloromethane (72 mL) at room temperature for 2 hrs. Water (30 mL) was added. The organic layer was separated and washed with brine (2 x30 mL). Evaporation of solvent followed by column chromatography (30% ethyl acetate/hexanes -50% ethyl acetate/hexanes) afforded methyl 12-((2S,3R)-1-(2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethylamino)-3-hydroxy-1-oxobutan-2-ylamino)-12-oxododecanoate **154** as a waxy yellow semi-solid (2.60g, 52.6%). $^1$HNMR (400MHz, acetone-d6, ppm): δ 7.51 (t, J = 5.5 Hz, 1H), 7.45-7.49 (m, 2H), 7.28-7.36 (m, 6H), 7.21 (tt, J = 7.2, 1.2 Hz, 1H), 7.08 (d, J = 8.1 Hz, 1H), 6.88 (dt, J = 8.9, 2.5 Hz, 4H), 4.39 (dd, J = 8.2, 3.0 Hz, 1H), 4.20-4.27 (m, 1H), 3.78 (s, 6H), 3.60 (s, 1H), 3.35-3.52 (m, 2H), 3.07-3.16 (m, 2H), 2.23-2.37 (m, 4H), 1.53-1.65 (m, 4H), 1.23-1.36 (m, 12H), 1.10 (d, J = 6.4 Hz, 3H).

**Scheme 24**




**Step 11. Preparation of Compound 164-1**

To a suspension of potassium t-butoxide (14.6 g, 130 mol) in THF (120 mL)/ether (360 mL) was added methyltriphenylphosphonium bromide (46.6 g,130 mmol). The mixture was refluxed for 2 hrs and then cooled to 0°C. *tert*-butyl 2-formylpyrrolidine-1-carboxylate (13.0g, 65.2 mmol) in ether (50 mL) was added dropwise. The reaction mixture was stirred at 0°C and then quenched by the addition of water (250 mL). The organic layer was separated and the aqueous was extracted with ether (250 mL). The combined extract was dried over $MgSO_4$. Evaporation of solvent, followed by column chromatography purification (5% ethyl acetae/hexanes) gave *tert*-butyl 3-vinylpyrrolidine-1-carboxylate **164-1** (11.5g, 89.4%) as a colorless liquid. GC-MS: *m/z*: 197 (2) ($M^+$), 141 (40), 124 (30), 57 (100).

**Step 12. Preparation of Compound 164-2**

To a mixture of *t*-BuOH (140 mL) and water (70 mL), was charged AD-mix-β (47.4 g) and methanesulfonamide (2.89 g, 30.4 mmol). The mixture was stirred at room temperature for 30 min and was then cooled to 0°C. *tert*-Butyl 3-vinylpyrrolidine-1-carboxylate **164-1** (6.00g, 30.4 mmol) was added. The reaction was stirred at room temperature overnight. The reaction mixture was cooled to 0°C. Sodium thiosulfate pentahydrate (96 g, 387 mmol) was added and the temperature was allowed to warm to room temperature. Water (700mL) was added and the mixture was extracted with ethyl acetate (500 mL). The extract was washed with water (2 x 50 mL) and brine (50 mL), and dried over $MgSO_4$. Evaporation of solvent, followed by column chromatography (2% methanol/dichloromethane - 7% methanol/dichloromethane) gave *tert*-butyl 3-(1,2-dihydroxyethyl)pyrrolidine-1-carboxylate **164-2** (5.4 g, 77%) as a light brown oil.

**Step 13. Preparation of Compound 164-3**

To a solution of *tert*-butyl 3-(1,2-dihydroxyethyl)pyrrolidine-1-carboxylate **164-2** (3.1g, 13.4 mmol) in ethanol (10 mL) was added 3N HCl (30 mL, 90 mmol). The reaction mixture was stirred at room temperature overnight. TLC indicated the completion of the reaction. Ethanol was evaporated. Toluene was added and evaporated. This was repeated three times to give 1-(pyrrolidin-3-yl)ethane-1,2-diol hydrochloride **164-3** (2.0g, 89%) as a brown oil. LC-MS (ESI): *m/z*: 132 (100), ($M + H^+$, free amine).

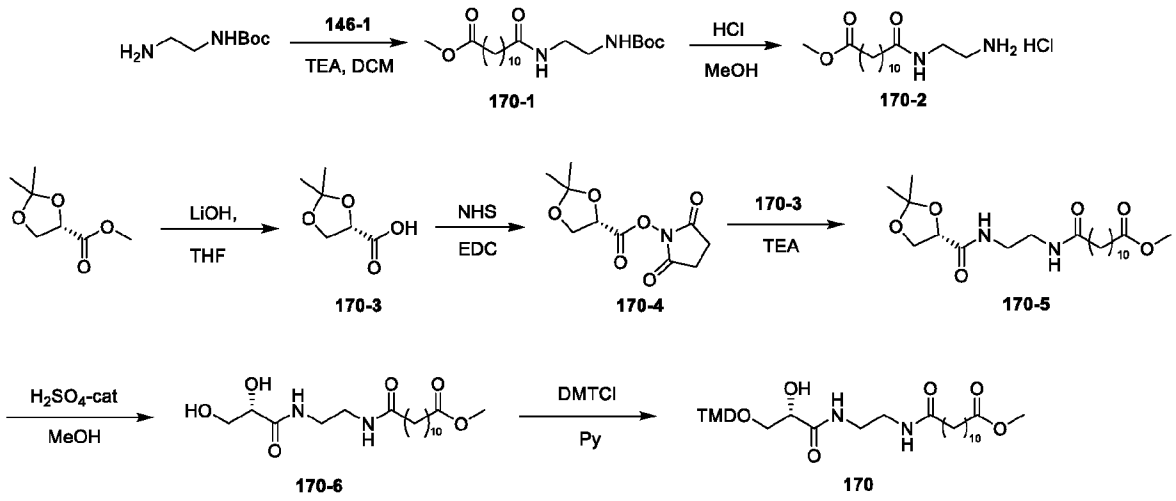
**Step 14 Preparation of Compound 164-4**

To a solution of 1-(pyrrolidin-3-yl)ethane-1,2-diol hydrochloride **164-2** (2.0g, 12 mmol) in water (30 mL) was added $NaHCO_3$ (3.7g, 44 mmol) by portion. Dioxane (20 mL) was then added. To the above solution was added 1-(2,5-dioxopyrrolidin-1-yl) 12-methyl dodecanedioate **146-1** (3.7g, 11 mmol) in dioxane (30 mL). The reaction mixture was stirred overnight. This was extracted with ethyl acetate (3 x100 mL). The combined extract was washed with 0.5N HCl (50 mL) and brine (50 mL), and dried over $MgSO_4$.

**Step 15. Preparation of Compound 164**

This substance was prepared from methyl 12-(3-(1,2-dihydroxyethyl)pyrrolidin-1-yl)-12-oxododecanoate **164-4** and 4,4-dimethoxytrityl chloride (1 eq) using the same procedure as described in the synthesis of 2-(2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)isoindoline-1,3-dione **138**. The product was purified by column chromatography (1.5% methanol/dichloromethane). Methyl 12-(3-(2-(bis(4-methoxyphenyl)(phenyl)methoxy)-1-hydroxyethyl)pyrrolidin-1-yl)-12-oxododecanoate **164** was obtained in 51% yield as a yellow oil. $^1$HNMR (400MHz, acetone-d6, ppm): δ 7.49-7.54 (m, 2H), 7.35-7.40 (m, 4H), 7.28-7.34 (m, 2H), 7.19-7.25 (m, 1H), 6.86-6.91 (m, 4H), 4.11-4.20 (m, 1H), 3.79 (s, 6H), 3.68-3.77 (m, 1H), 3.60 (s, 3H), 3.29-3.59 (m, 3H), 3.06-3.20 (m, 3H), 2.33-2.55 (m, 1H), 2.29 (t, J = 7.4 Hz, 2H), 2.19 (t, J = 7.6 Hz, 2H), 1.65-2.0 (m, 2H), 1.51-1.62 (m, 4H), 1.26-1.35 (m, 12H).

**Scheme 25**




**Step 16. Preparation of Compound 170-1**

To a solution of *tert*-butyl 2-aminoethylcarbamate (2.88g, 18.0 mmol) and triethylamine (2.98g, 29.4 mmol) in dichloromethane (100 mL), was added 1-(2,5-dioxopyrrolidin-1-yl) 12-methyl dodecanedioate (**146-1**) (5.09g, 14.9 mmol) in dichloromethane (50 mL) dropwise at room temperature. The reaction mixture was stirred overnight and TLC indicated the completion of the reaction. 100 mL brine was added and the organic layer was separated. The organic layer was washed with 0.5N HCl (150 mL), brine ( 2 x 100 mL) and dried over $MgSO_4$. Evaporation of solvent gave pure methyl 12-(2-(*tert*-butoxycarbonylamino)ethylamino)-12-oxododecanoate **170-1** (5.85g 100%) as a white solid.

**Step 17. Preparation of Compound 170-2**

To a solution of 12-(2-(*tert*-butoxycarbonylamino)ethylamino)-12-oxododecanoate **170-1** (5.55g, 14.4 mmol) in methanol (100 mL) at 0°C, was added thionyl chloride (3.3 mL, 45.5 mmol) dropwise. The reaction was then stirred at room temperature overnight. TLC indicated the completion of the reaction. The solvent and volatile organics were evaporated. The residue was then co-evaporated with heptanes twice to give methyl 12-(2-aminoethylamino)-12-oxododecanoate hydrochloride **170-2** quantitatively as a white solid. LC-MS (ESI): *m/z*: 287 (100), ($M + H^+$, free amine).

**Step 18. Preparation of Compound 170-3**

(-)-Methyl (S)-2,2-dimethyl-1,3-dioxolane-4-carboxylate (5.01g, 31.2 mmol) and $LiOH{\cdot}H_2O$ (2.55g, 60.8 mmol) in THF (50 mL) and water (50 mL) was stirred overnight. TLC

indicated the completion of the reaction. THF was evaporated and the aqueous was acidified with 1N HCl to pH = 1. This was extracted with ethyl acetate ( 5 x 50 mL). The combined extract was dried over $MgSO_4$. Evaporation of solvent gave (S)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid **170-3** (2.93g, 64.3%) as a light yellow liquid.

**Step 19. Preparation of Compound 170-4**

Compound **170-4** was synthesized from (S)-2,2-dimethyl-1,3-dioxolane-4-carboxylic acid **170-3** and N-hydroxysuccinimide in 86% yield, using the same procedure as described in the synthesis of 1-(2,5-dioxopyrrolidin-1-yl) 12-methyl dodecanedioate **146-1**. (S)-2,5-Dioxopyrrolidin-1-yl 2,2-dimethyl-1,3-dioxolane-4-carboxylate **170-4** was obtained in 86% yield as a white solid.

**Step 20. Preparation of Compound 170-5**

To a suspension of methyl 12-(2-aminoethylamino)-12-oxododecanoate hydrochloride **170-2** (14.4 mmol) and (S)-2,5-dioxopyrrolidin-1-yl 2,2-dimethyl-1,3-dioxolane-4-carboxylate **170-4** (3.80g, 15.6 mmol) in dichloromethane (100 mL) was added triethylamine (6 mL, 43.0 mmol) in dichloromethane (25 mL) over 4 hrs at 0°C. The reaction mixture was then stirred at room temperature overnight. LC-MS indicated that the starting material **170-2** was completely converted. The organic layer was washed with brine (50 mL), 1N HCl (50 mL), brine (50 mL), dried over $MgSO_4$ and concentrated to dryness to afford (S)-methyl 12-(2-(2,2-dimethyl-1,3-dioxolane-4-carboxamido)ethylamino)-12-oxododecanoate **170-5** (5.93g, 99.3%) as a white solid.

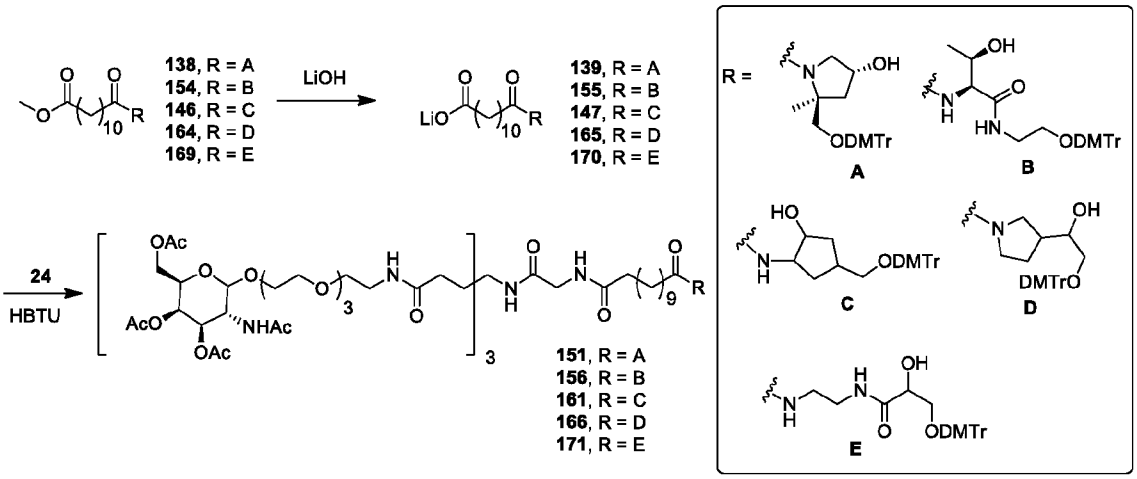
**Step 21. Preparation of Compound 170-6**

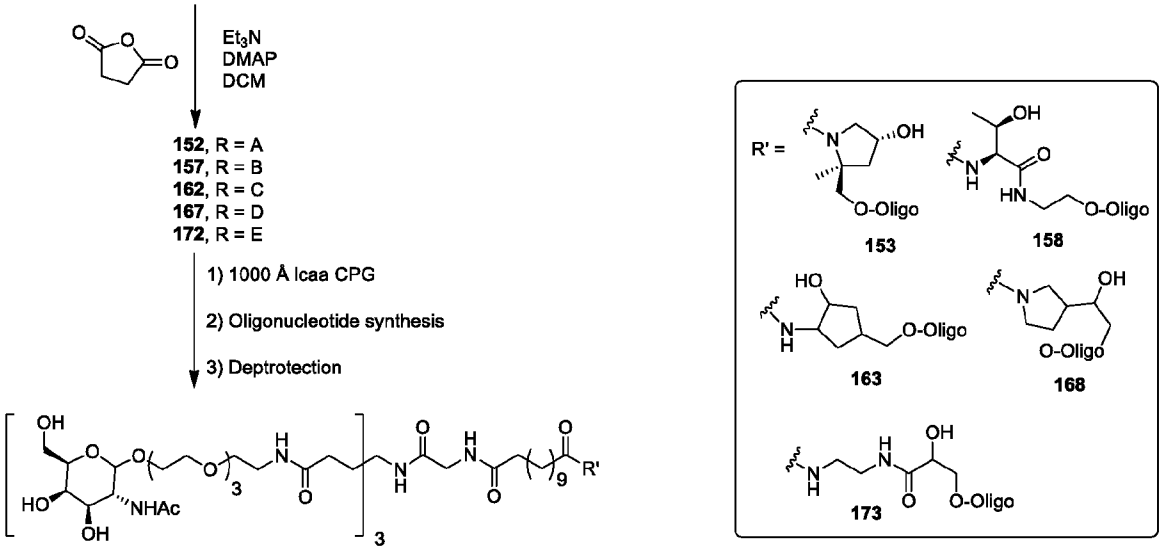
To a solution of (S)-methyl 12-(2-(2,2-dimethyl-1,3-dioxolane-4-carboxamido)ethylamino)-12-oxododecanoate **170-5** (5.93g, 14.3 mmol) was added one drop of concentrated sulfuric acid. This was refluxed for 6 hrs and then cooled to room temperature. The solid was collected through filtration and washed twice with cold methanol. The solid was dried in the air (3.32g). The second crop (0.42g) was obtained from the mother liquid to give (S)-methyl 12-(2-(2,3-dihydroxypropanamido)ethylamino)-12-oxododecanoate **170-6** (3.74g in total, 69.4%) as a white crystal. LC-MS (ESI): *m/z*: 375 (100), $(M + H^+)$. $^1$HNMR (400MHz, DMSO-d6, ppm): δ 7.79 (br, 2H), 5.49 (d, J = 5.3 Hz, 1H), 4.66 (t, J = 5.8 Hz, 1H), 3.83-3.88 (m, 1H), 3.55-3.61 (m, 4H), 3.41-3.47 (m, 1H), 3.05-3.15 (m, 4H), 2.29 (t, J = 7.4 Hz, 2H), 2.03 (t, J = 7.6 Hz, 2H), 1.42-1.52 (m, 4H), 1.18-1.29 (m, 12H).

**Step 22. Preparation of Compound 170**

To a solution of (S)-methyl 12-(2-(2,3-dihydroxypropanamido)ethylamino)-12-oxododecanoate **170-6** (2.99g, 7.99 mmol) in dry pyridine (57.5 mL) under nitrogen, was added 4,4'-dimethoxytrityl chloride (2.84g, 8.38 mmol) in one portion. The reaction was stirred at room temperature for two days. Methanol (5 mL) was added to quench the reaction. Pyridine was evaporated. Toluene was added and then evaporated. This was repeated three times. Water (100 mL) was added and this was extracted with ethyl acetate (5 x 250 mL). The extracts were combined and dried over MgSO4. Evaporation of solvent, followed by column chromatography (1%methanol/dichloromethane-3% methanol/dichloromethane) gave (S)-methyl 12-(2-(3-(bis(4-methoxyphenyl)(phenyl)methoxy)-2-hydroxypropanamido)ethylamino)-12-oxododecanoate **170** (1.70g, 31.4%) as a viscous oil. $^1$HNMR (400MHz, acetone-d6, ppm): δ 7.64-7.70 (br, 1H), 7.47-7.51 (m, 2H), 7.33-7.37 (m, 4H), 7.26-7.32 (m, 2H), 7.20 (dt, J = 7.3, 2.1 Hz, 1H), 7.11 (br, 1H), 6.86 (d, J = 8.7 Hz, 4H), 4.84 (br, 1H), 4.21 (dd, J = 5.1, 3.8 Hz, 1H), 3.78 (s, 6H), 3.60 (s, 1H), 3.25-3.42 (m, 6H), 2.28 (t, J = 7.4 Hz, 2H), 1.48-1.62 (m, 4H), 1.21-1.34 (m, 12H).

**Scheme 26.**






**Step 23. Preparation of compounds 139, 155, 160, 165 and 170**

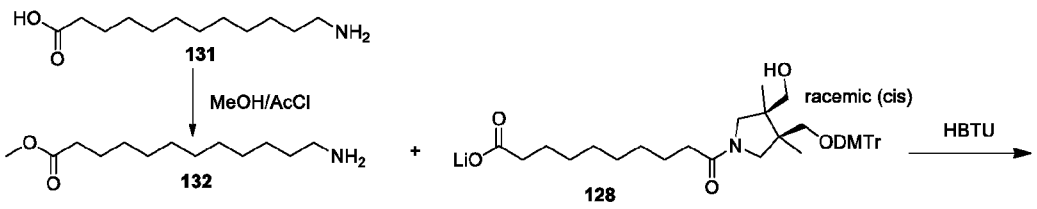
Compounds **139, 155**, **160**, **165** and **170** were prepared from compounds **138, 154**, **159**, **164** and **169** using an identical procedure to that used for compound **18**.

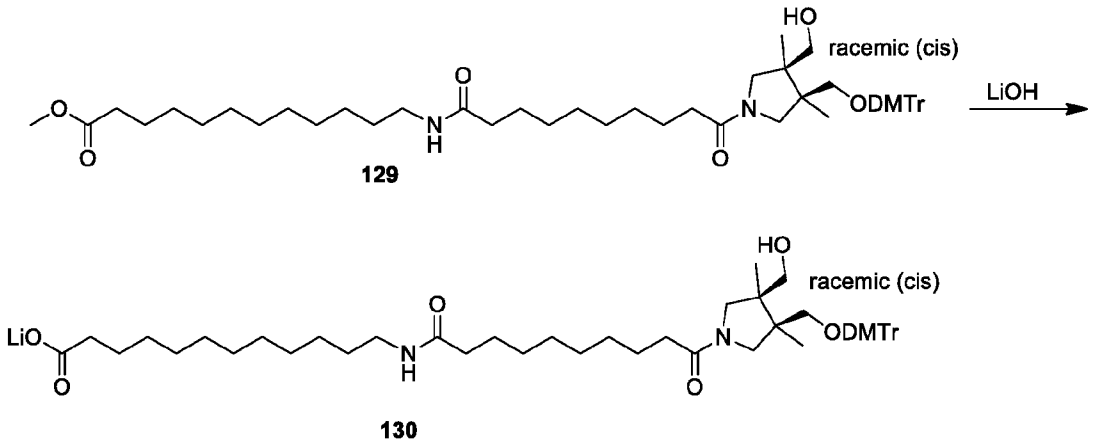
**Step 24. Preparation of conjugates 153, 158, 163, 168 and 173**

Conjugates **153, 158, 163, 168 and 173** were prepared from compound **139**, **154**, **159**, **164** and **169** using an identical procedure to that used for compound **1**.

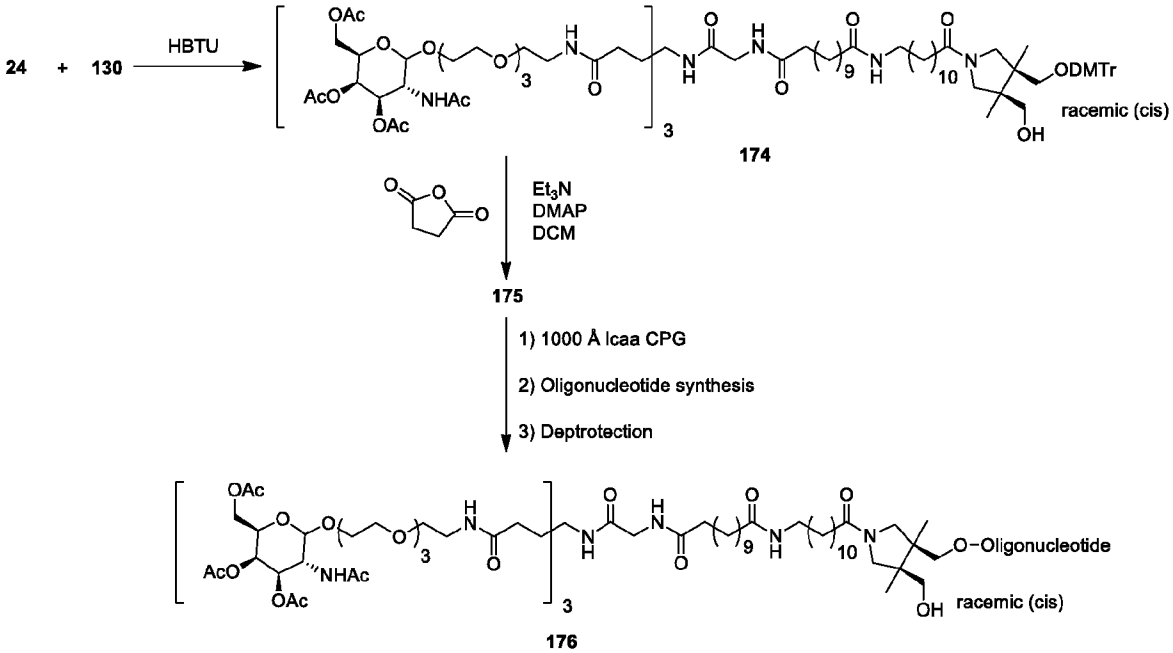
## Example 12. Synthesis of conjugate 176

**Scheme 27.**






**Scheme 28.**




**Step. 1. Preparation of methyl 12-aminododecanoate 132**

12-aminoundecanoic acid (**131**) (10g, 4.64 mmol) was stirred in MeOH at RT. Acetyl chloride (856µL, 12 mmol) was added dropwise and the reaction stirred for 1.5 hr. The solvent was removed in-vacuo, the residue taken up in MTBE and chilled in the fridge overnight. The resultant precipitate was collected by filtration, washed with ice cold MTBE and dried under high vacuum to afford methyl 12-aminododecanoate **132**.

**Step 2. Preparation of Racemic (cis) Methyl 12-(12-(10-(3-((bis(4-methoxyphenyl)-(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanamido)dodecanamido)dodecanoate 129**

Lithium racemic (cis) 10-(3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate (**128**) (2g, 3.1 mmol), of methyl 12-aminododecanoate (**132**) (778 mg, 3.1 mmol), HBTU (1.2 g, 3.1 mmol) and TEA (1.4 mL, 10 mmol) were stirred in DCM at RT O/N. The precipitate was removed by filtration, the filtrate concentrated in-vacuo and the residue purified by column chromatography (5% MeOH, DCM). TLC showed two close running spots with identical mass that were assigned as geometric isomers and pooled together to give of Methyl 12-(12-(10-((3R,4S)-3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanamido)dodecanamido)dodecanoate (**129**) in quantitative fashion.

**Step 3. Preparation of Racemic (cis) Lithium 12-(12-(10-(-3-((bis(4-methoxyphenyl)(phenyl)-methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanamido)-dodecanamido)dodecanoate 130**

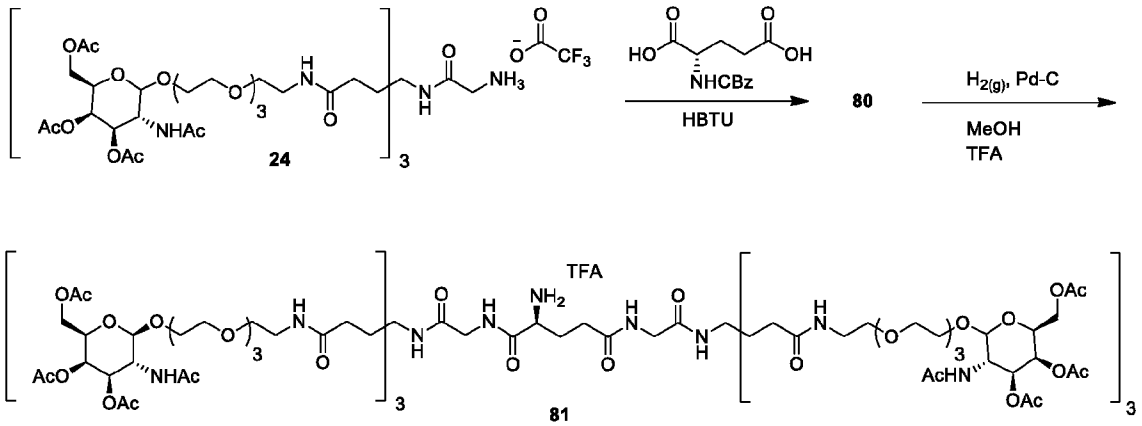
Racemic (cis) methyl 12-(12-(10-(3-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanamido)dodecanamido)dodecanoate (**129**) (3.1 mmol) was stirred in THF:$H_2O$ (50:50) with LiOH (88 mg, 3.7 mmol) at RT O/N. Reaction was confirmed by TLC and the THF removed in-vacuo. The aqueous solution was frozen in liquid $N_2$ and lyophilized for 48 hours to give racemic (cis) Lithium 12-(12-(10-(3-((bis(4-methoxyphenyl)(phenyl)-methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanamido)-dodecanamido)dodecanoate **130** quantitatively.

**Step 4. Preparation of conjugate 176**

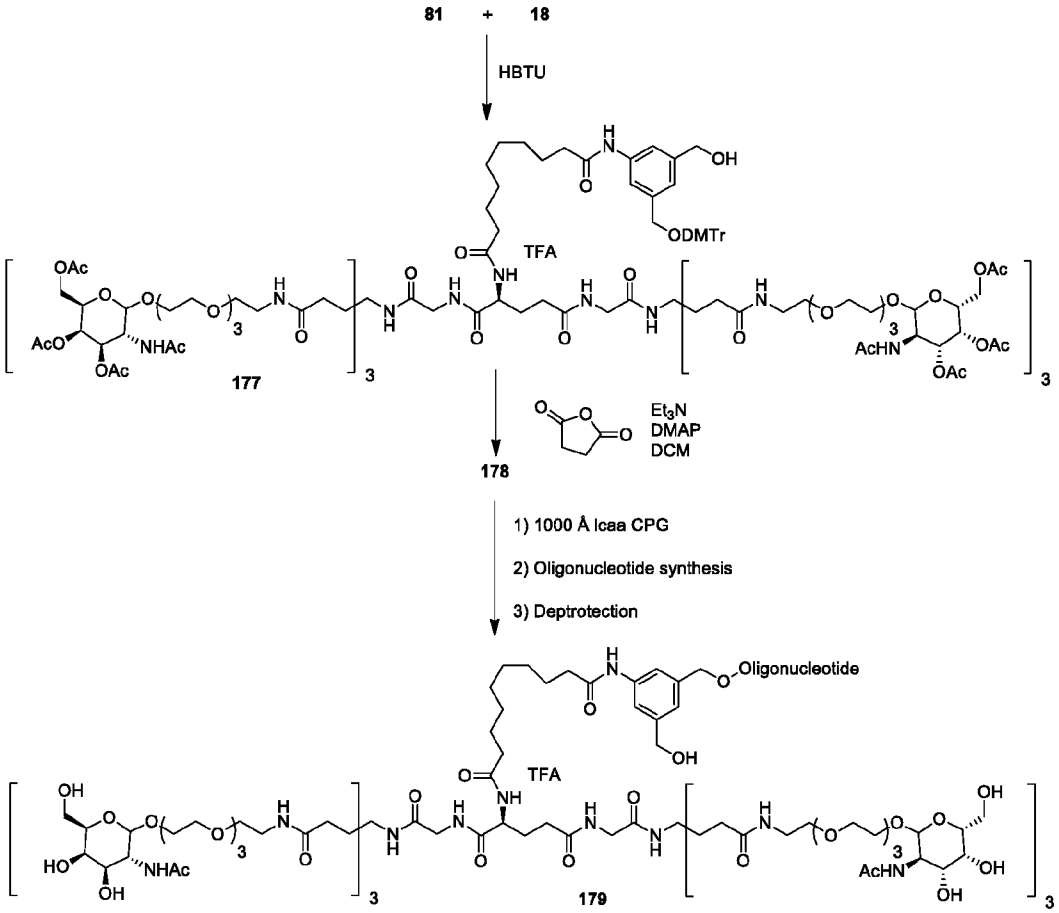
Conjugate **176** was prepared from compounds **24** and **130** using an identical procedure to that used for compound **1**.

## Example 13. Synthesis of conjugate 179

**Scheme 29.**




**Scheme 30.**



**Step 1. Preparation of compound 80**

Compound **24** (2g, 0.86 mmol), N-carbobenzoxy-L-glutamic acid (120 mg, 0.43 mmol), HBTU (326 mg, 0.86 mmol) and TEA (353 μL, 2.6 mmol) were stirred in DCM at RT O/N. The mixture was concentrated in-vacuo and purified by column chromatography to give compound **80** (2.88 g, 83%).

**Step 2. Preparation of compound 81**

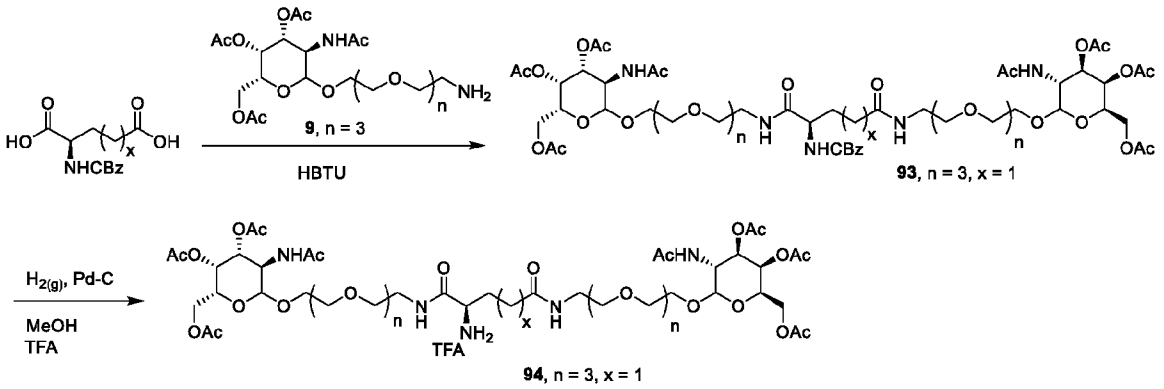
Compound **81** was prepared from compounds **80** (670 mg, 0.17 mmol) using an identical procedure to that used for compound **14**. The compound was used crude in subsequent reactions and the yield taken as quantitative.

**Step 3. Preparation of conjugate179**

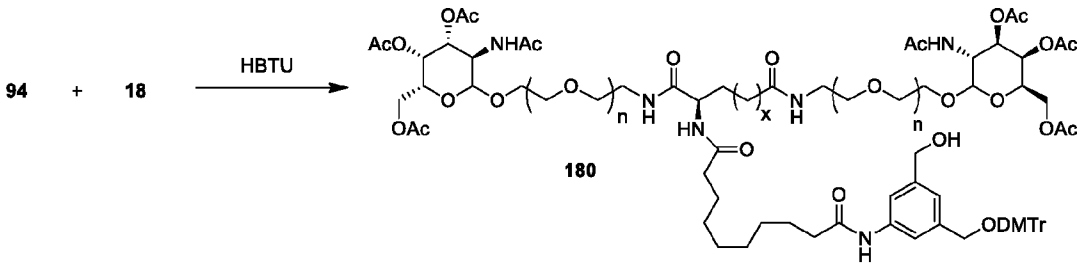
Conjugate **179** was prepared from compounds **18** and **81** using an identical procedure to that used for compound **1**.

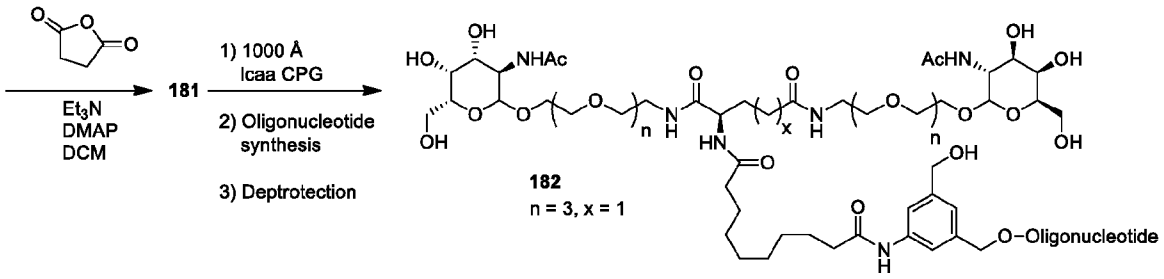
**Example 14. Synthesis of conjugate 182**

**Scheme 31.**




**Scheme 32.**






**Step 1. Preparation of compound 93**

Compound **93** was prepared from (2-oxo-2-phenyl-1$\lambda^2$-ethyl)-D-glutamic acid (2.25 g, 8.1 mmol) and **9** (13 g, 21 mmol) using an identical procedure to that used for compound **89**. Yield: 11.2 g.

**Step 2. Preparation of compound 94**

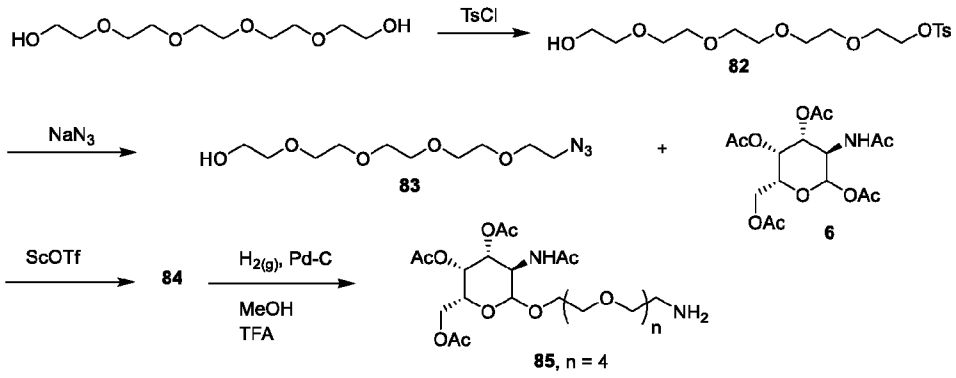
Compound **94** was prepared from compound **93** (11.1 g) using an identical procedure to that used for compound **90**. Yield: 10.2 g

**Step 3. Preparation of conjugate 182**

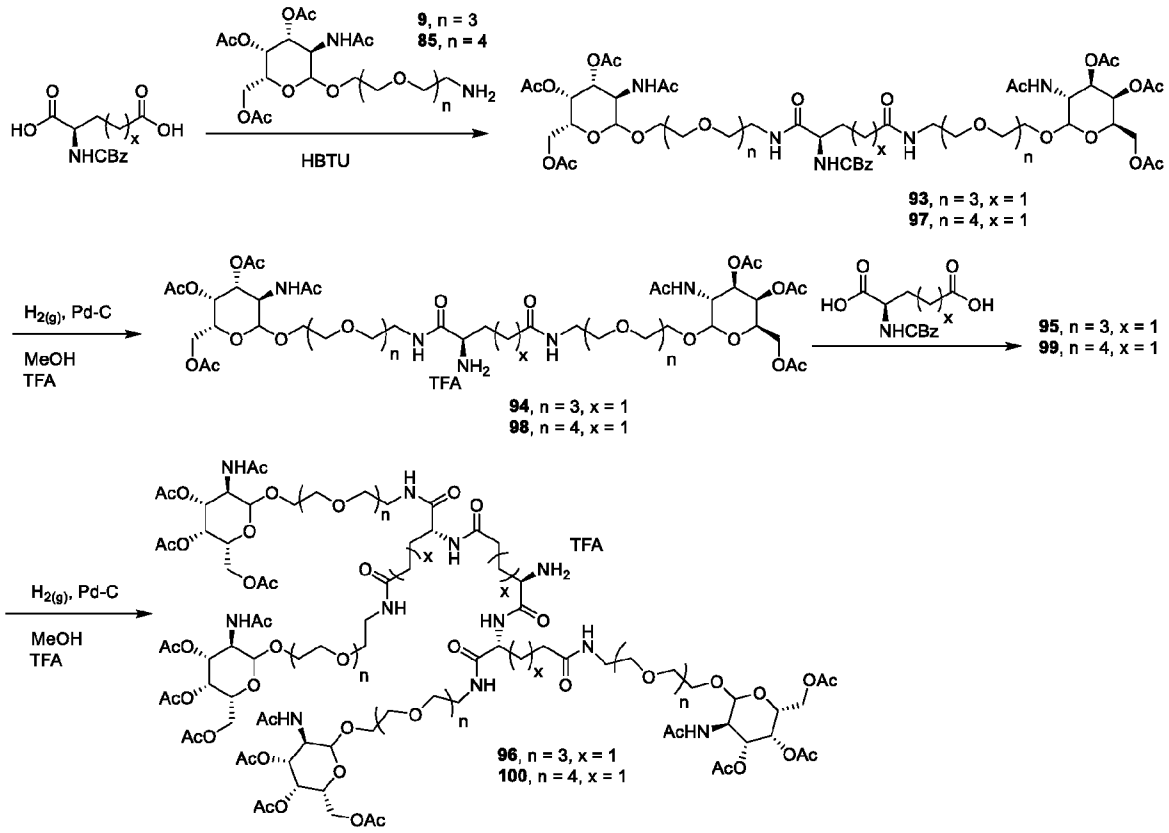
Conjugate **182** was prepared from compounds **18** and **94** using an identical procedure to that used for compound **1**.

## Example 15. Synthesis of conjugates 185 and 188

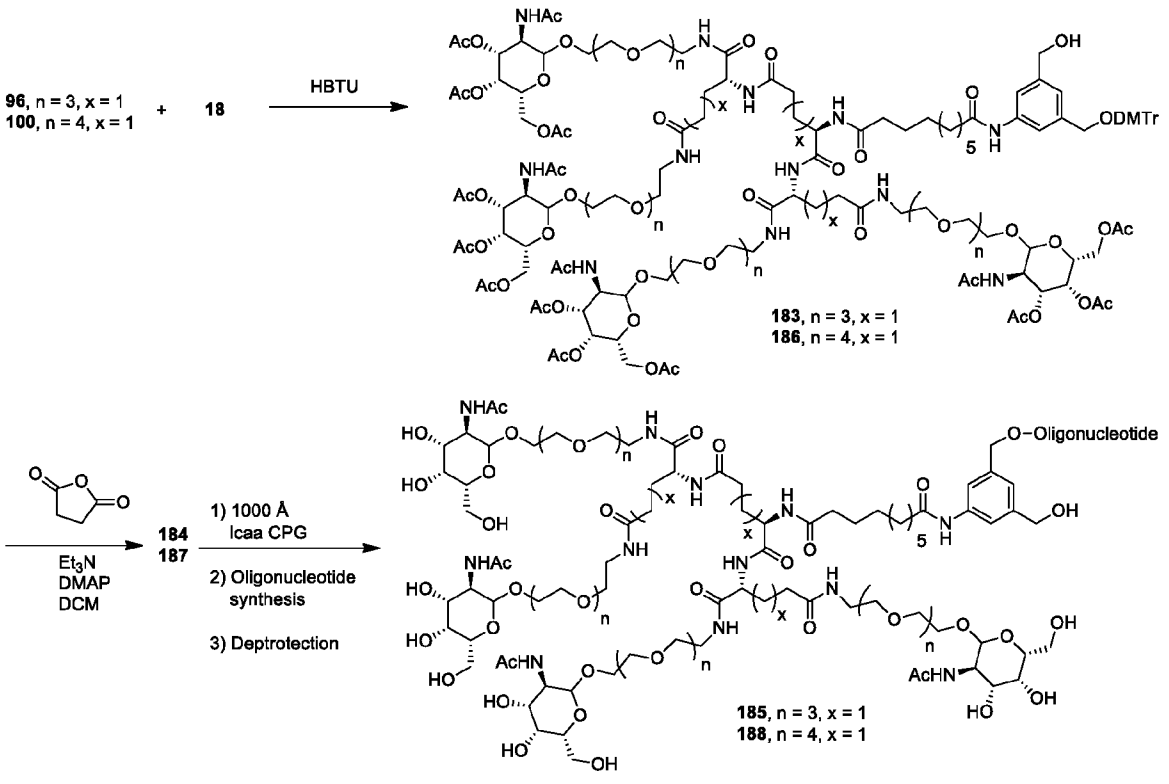
**Scheme 33.**



**Scheme 34.**




**Scheme 35.**



**Step 1. Preparation of 14-Hydroxy-3,6,9,12-tetraoxatetradecyl 4-methylbenzenesulfonate 82**

A solution of pentaethylene glycol (35g, 147mmol), TEA (41mL, 294mmol) and trimethylamine-HCl (1.4g, 14.7mmol) in $CH_2Cl_2$ (600mL) was treated with tosyl chloride (29.4g, 154mmol). After stirring (18h) the reaction mixture was washed with $H_2O$-brine (1:1), dried ($MgSO_4$), filtered, concentrated and subjected to chromatography to yield **82** (24.6g, 43%) as a pale yellow oil. Rf 0.8 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 2. 14-azido-3,6,9,12-tetraoxatetradecan-1-ol 83**

14-azido-3,6,9,12-tetraoxatetradecan-1-ol **(83)** was prepared from **82** (24.6g, 62.7mmol) and sodium azide (7.13g, 110mmol) using an identical procedure to that used for compound **4**. Yield: 14.8g, 90%.

**Step 3. Preparation of compound 84**

A solution of GalNAc **6** (12.2g, 31.4mmol) and HO-PEG-$N_3$ **83** (9.2g, 35mmol) in 1,2-dichloroethane (150mL) was treated with $Sc(OTf)_3$ (771mg, 1.6mmol). After stirring (85°C, 2hr) the reaction was cooled (RT), quenched by the addition of TEA (40mL) and concentrated. The crude material was subjected to chromatography to yield **84** (11.16g, 60%) as a pale yellow foam. Rf 0.7 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 4. Preparation of compound 85**

A solution of **84** (11.16g, 18.8mmol) and Pd/C (1.1g, 10% - wet support) in EtOAc (120mL) was treated with TFA (4.32mL, 56.5mmol) and purged with $H_2$. After stirring vigorously (4.5h) the reaction was purged with $N_2$, filtered through Celite and concentrated. The crude material was subjected to chromatography to yield **85** (5.77g, 45%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 5. Preparation of compound 95**

Compound **95** was prepared from (2-oxo-2-phenyl-1$\lambda^2$-ethyl)-D-glutamic acid **(**1.04 g, 3.7 mmol) and compound **94 (**10.2 g) using an identical procedure to that used for compound **91**. Yield: 7.2 g

**Step 6. Preparation of compound 96**

Compound **96** was prepared from compound **95** (11.1 g) using an identical procedure to that used for compound **92**. Yield: 6.5 g

**Step 7. Preparation of compound 97**

Compound **97** was prepared from (2-oxo-2-phenyl-1$\lambda^2$-ethyl)-D-glutamic acid (2g, 7.1mmol) and **85** (12.1g, 17.8mmol) using an identical procedure to that used for compound **89**. Yield: 10g, quantitative.

**Step 8. Preparation of compound 98**

Compound **98** was prepared from compound **97** (10g, 7.2mmol) using an identical procedure to that used for compound **90**. Yield: 3.5g, 36%.

**Step 9. Preparation of compound 99**

Compound **99** was prepared quantitatively from (2-oxo-2-phenyl-1$\lambda^2$-ethyl)-D-glutamic acid (350 mg, 1.25 mmol) and compound **98** (2.86 mg, 2.5mmol) using an identical procedure to that used for compound **91**.

**Step 10. Preparation of compound 100**

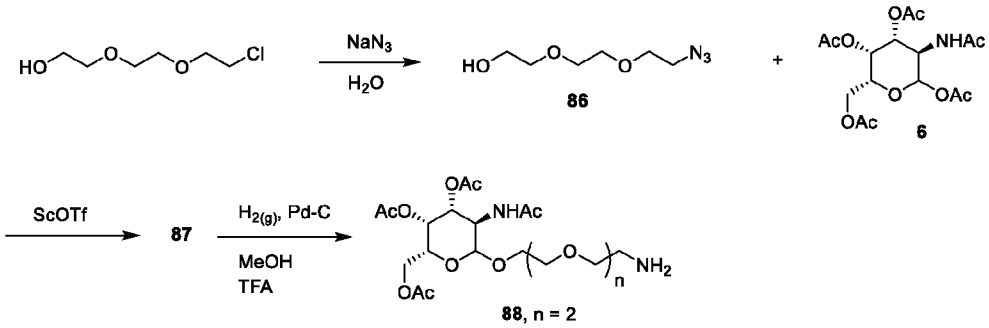
Compound **100** was prepared quantitatively from compound **99** (3.2 g, 1.25 mmol) using an identical procedure to that used for compound **92.**

**Step 11. Preparation of conjugates 185 and 188**

Conjugate **185** and **188** were prepared from compounds **18** and **96** or **18** and **100** using an identical procedure to that used for compound **1**.

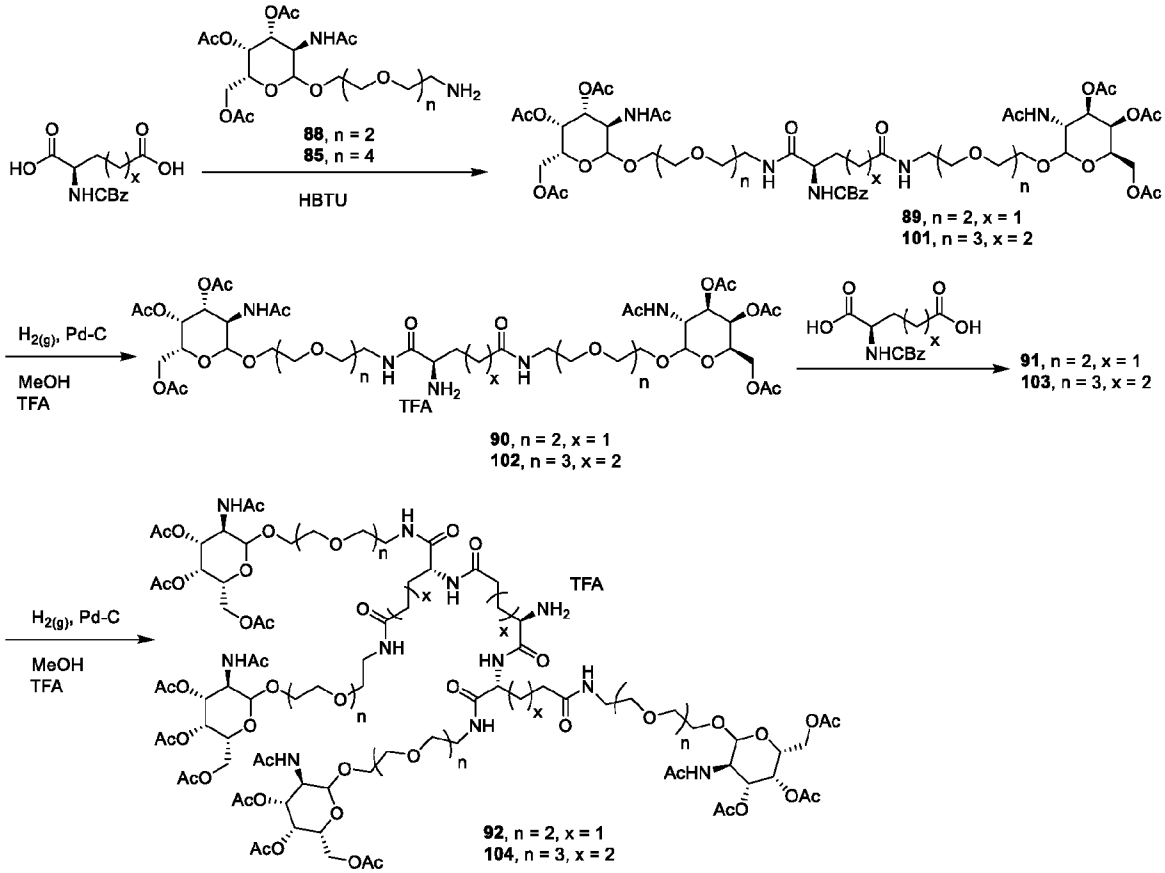
## Example 16. Synthesis of conjugates 191, 194, 197 and 200

### Scheme 36

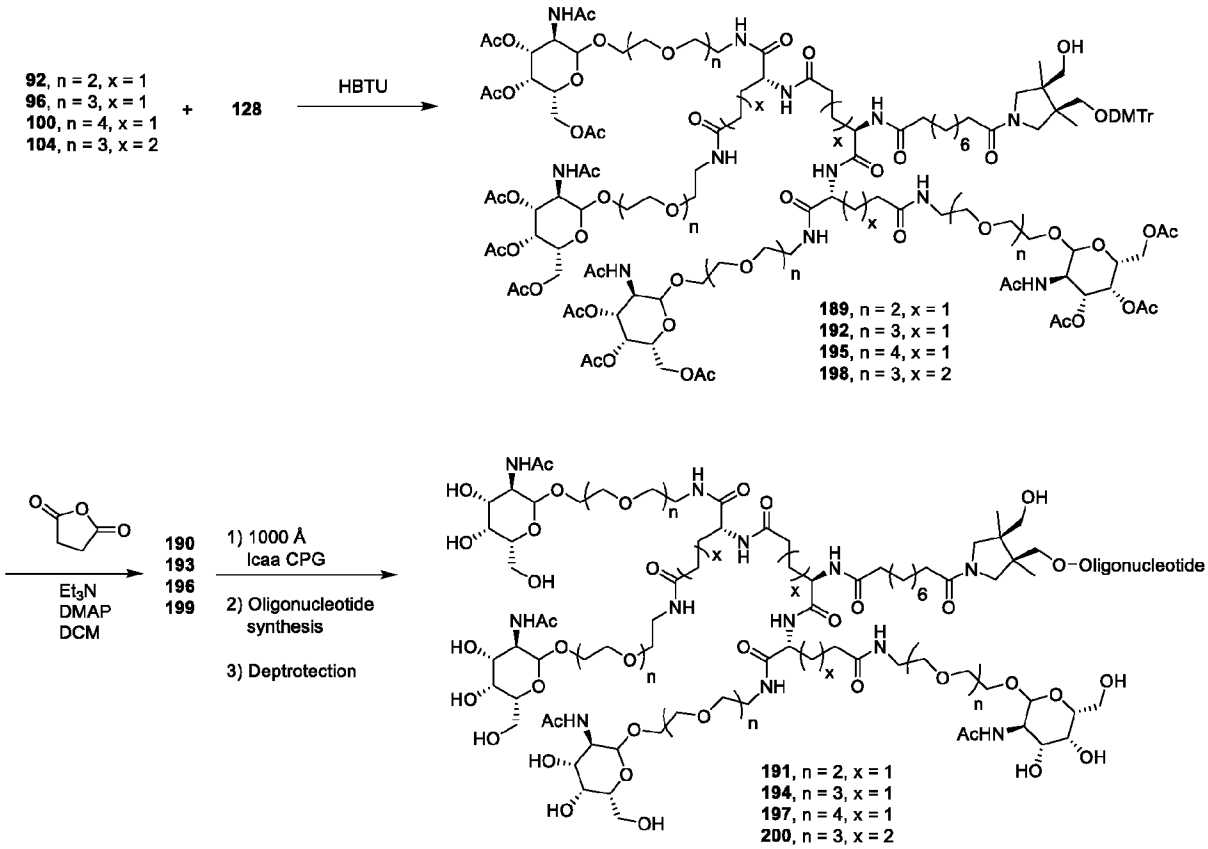



5

### Scheme 37.



**Scheme 38.**




**Step 1. Preparation of 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol 86**

To a solution of 2-(2-(2-chloroethoxy)ethoxy)ethan-1-ol (13 g, 77 mmol) in water (200 mL) was added sodium azide (10 g, 154 mmol). The reaction was heated to 100°C for 18 hours. The reaction was cooled to room temperature and poured into a 1L separatory funnel and extracted with dichloromethane (3 x 200 mL). The combine dichloromethane extracts were dried on magnesium sulfate, filtered and concentrated to dryness to afford 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol as a colorless oil (11.7 g)

**Step 2. Preparation of compound 87**

Compound **87** was prepared from **86** (4.95g, 28.3mmol) and **6** (10g, 25.7mmol) using an identical procedure to that used for compound **84**. Yield: 10g, 77%.

**Step 3. Preparation of compound 88**

Compound **88** was prepared from **87** (10g, 19.8mmol) using an identical procedure to that used for compound **85**. Yield: 7.63g, 65%.

**Step 4. Preparation of compound 89**

A solution of **88** (2g, 3.38mmol) and Z-glutamic acid (427mg, 1.52mmol) in $CH_2Cl_2$ (50mL) was treated with HBTU (1.41g, 3.7mmol) and Hünig's base (1.77mL, 10.1mmol). After stirring (18h) the mixture was concentrated and subjected to chromatography to yield **89** (871mg, 48%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 5. Preparation of compound 90**

A solution of **89** (870mg, 0.72mmol) and Pd/C (90mg, 10% - wet support) in EtOAc (10mL) was treated with TFA (84µL, 1.1mmol) and purged with $H_2$. After stirring vigorously (2h) the reaction was purged with $N_2$, filtered through Celite and concentrated. The crude material was used without further processing and yielded **90** (850mg, quantitative) as a colorless foam. Rf 0.25 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 6. Preparation of compound 91**

A solution of **90** (850mg, 0.72mmol) and Z-glutamic acid (91mg, 0.32mmol) in $CH_2Cl_2$ (10mL) was treated with HBTU (300mg, 0.79mmol) and Hünig's base (502µL, 2.9mmol). After stirring (1.5h) the mixture diluted with $CH_2Cl_2$ and washed with $NaHCO_3$ (Sat. Aq.), dried ($MgSO_4$), filtered and concentrated. The crude material was subjected to chromatography to yield **91** (590mg, 76%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 7. Preparation of compound 92**

A solution of **91** (590mg, 0.25mmol) and Pd/C (100mg, 10% - wet support) in $CH_3OH$ (30mL) was treated with TFA (29µL, 0.37mmol) and purged with $H_2$. After stirring (3h) the mixture was purged with $N_2$, then filtered through Celite and concentrated. The crude material was used without further processing and yielded **92** (600mg, quantitative) as a colorless foam. Rf 0.1 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 8. Preparation of compound 101**

Compound **101** was prepared from (R)-2-((2-oxo-2-phenyl-1l2-ethyl)amino)hexanedioic acid (2.51g, 8.6 mmol) and **9** (11g, 17.2 mmol) using an identical procedure to that used for compound **89**. Yield: 4.2 g, 37%

**Step 9. Preparation of compound 102**

Compound **102** was prepared from compound **101** (4.2g, 3.2 mmol) using an identical procedure to that used for compound **90**. Yield: 2.1 g, 47%

**Step 10. Preparation of compound 103**

Compound **103** was prepared from (R)-2-((2-oxo-2-phenyl-1l2-ethyl)amino)hexanedioic acid (265 mg, 0.9 mmol) and compound **102** (2.1 g, 1.8 mmol) using an identical procedure to that used for compound **91**. Yield: (560 mg, 24 %)

**Step 11. Preparation of compound 104**

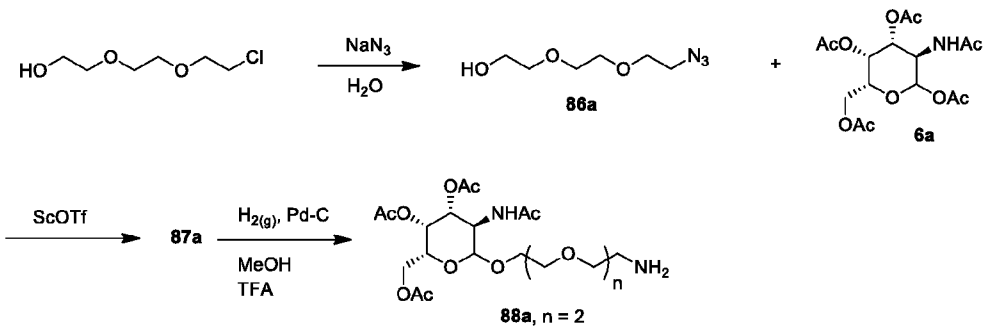
Compound **104** was prepared quantitatively from compound **103** (560 mg) using an identical procedure to that used for compound **92**. The compound was used without purification.

**Step 12. Preparation of conjugates 191, 194, 197 and 200**

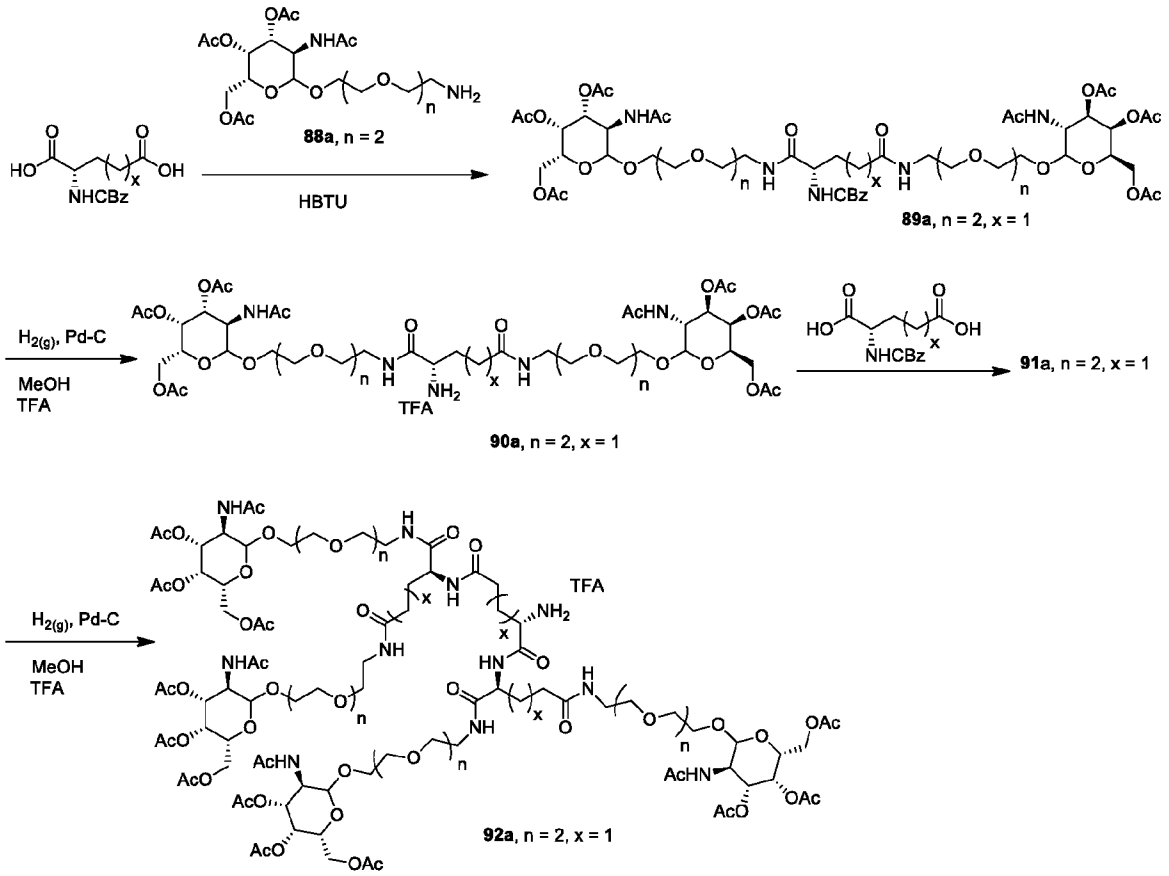
Conjugates **191, 194, 197** and **200** were prepared from compound **128** and **92, 96, 100** or **104** using an identical procedure to that used for compound **1**.

**Example 16a. Synthesis of conjugates 191a**

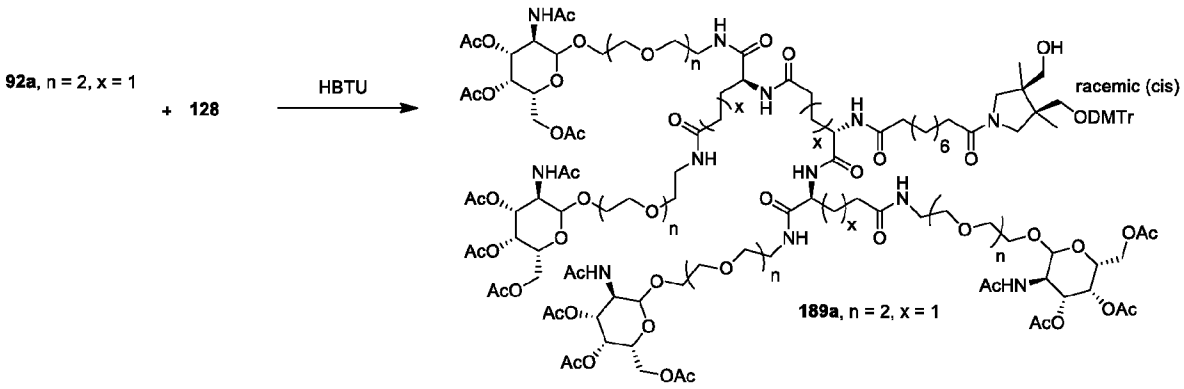
**Scheme 36a**

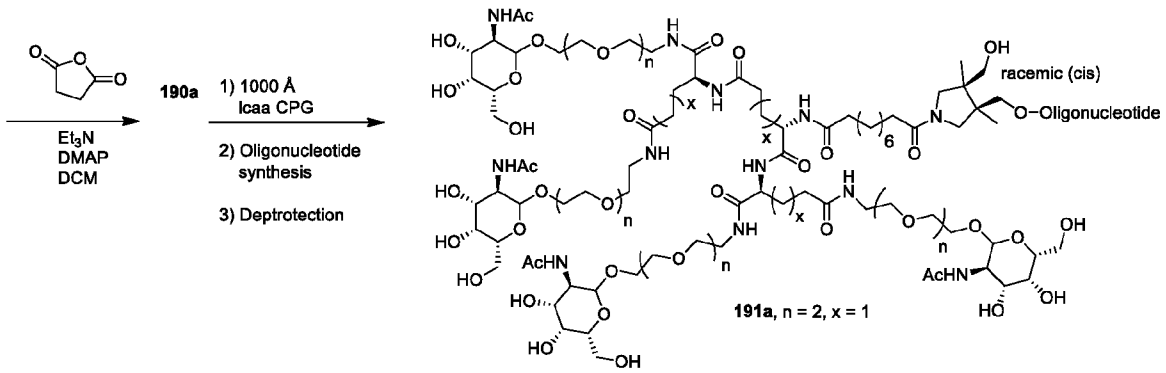


**Scheme 37a.**




**Scheme 38a.**






**Step 1. Preparation of 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol 86a**

To a solution of 2-(2-(2-chloroethoxy)ethoxy)ethan-1-ol (13 g, 77 mmol) in water (200 mL) was added sodium azide (10 g, 154 mmol). The reaction was heated to 100°C for 18 hours. The reaction was cooled to room temperature and poured into a 1L separatory funnel and extracted with dichloromethane (3 x 200 mL). The combine dichloromethane extracts were dried on magnesium sulfate, filtered and concentrated to dryness to afford 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol as a colorless oil (11.7 g).

**Step 2. Preparation of compound 87a**

Compound **87a** was prepared from **86a** (4.95g, 28.3mmol) and **6a** (10g, 25.7mmol) using an identical procedure to that used for compound **84**. Yield: 10g, 77%.

**Step 3. Preparation of compound 88a**

Compound **88a** was prepared from **87a** (10g, 19.8mmol) using an identical procedure to that used for compound **85**. Yield: 7.63g, 65%.

**Step 4. Preparation of compound 89a**

A solution of **88a** (2g, 3.38mmol) and Z—L-glutamic acid (427mg, 1.52mmol) in $CH_2Cl_2$ (50mL) was treated with HBTU (1.41g, 3.7mmol) and Hünig's base (1.77mL, 10.1mmol). After stirring (18h) the mixture was concentrated and subjected to chromatography to yield **89a** (871mg, 48%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 5. Preparation of compound 90a**

A solution of **89a** (870mg, 0.72mmol) and Pd/C (90mg, 10% - wet support) in EtOAc (10mL) was treated with TFA (84µL, 1.1mmol) and purged with $H_2$. After stirring vigorously (2h) the reaction was purged with $N_2$, filtered through Celite and concentrated. The crude

material was used without further processing and yielded **90a** (850mg, quantitative) as a colorless foam. Rf 0.25 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 6. Preparation of compound 91a**

A solution of **90a** (850mg, 0.72mmol) and Z-glutamic acid (91mg, 0.32mmol) in $CH_2Cl_2$ (10mL) was treated with HBTU (300mg, 0.79mmol) and Hünig's base (502µL, 2.9mmol). After stirring (1.5h) the mixture diluted with $CH_2Cl_2$ and washed with $NaHCO_3$ (Sat. Aq.), dried ($MgSO_4$), filtered and concentrated. The crude material was subjected to chromatography to yield **91a** (590mg, 76%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 7. Preparation of compound 92a**

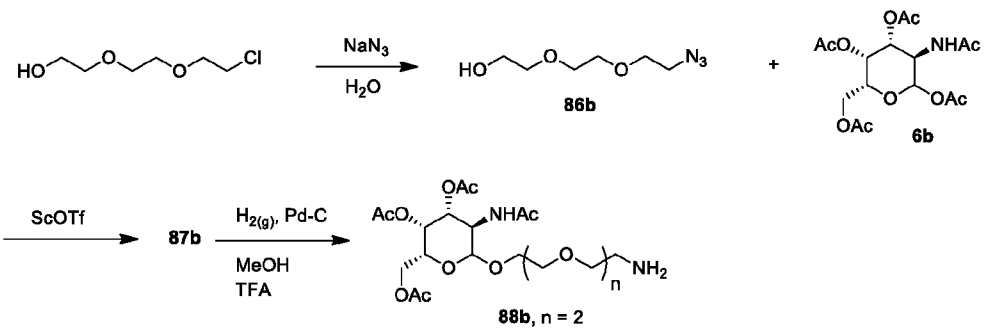
A solution of **91a** (590mg, 0.25mmol) and Pd/C (100mg, 10% - wet support) in $CH_3OH$ (30mL) was treated with TFA (29µL, 0.37mmol) and purged with $H_2$. After stirring (3h) the mixture was purged with $N_2$, then filtered through Celite and concentrated. The crude material was used without further processing and yielded **92a** (600mg, quantitative) as a colorless foam. Rf 0.1 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 8. Preparation of conjugate 191a,**

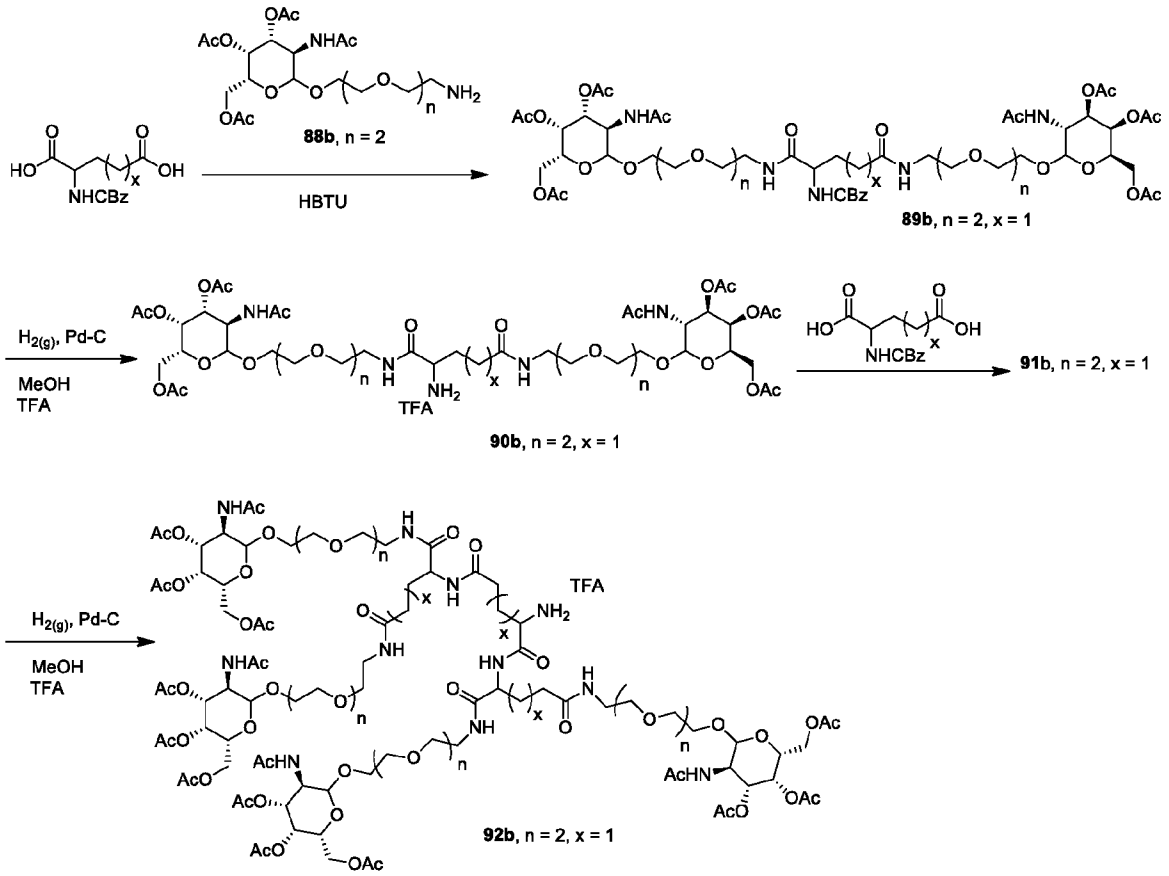
Conjugate **191a** was prepared from compound **128** and compound **92a** using an identical procedure to that used for compound **1**.

**Example 16b. Synthesis of conjugates 191b**

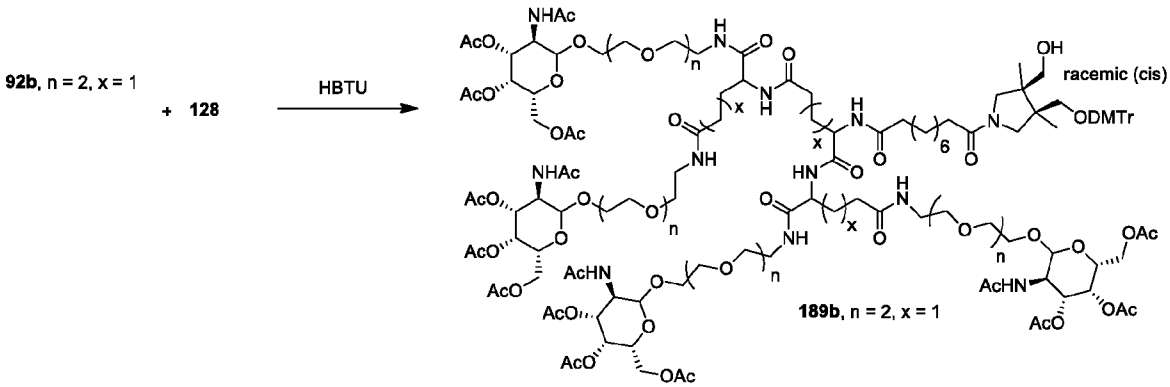
**Scheme 36b**

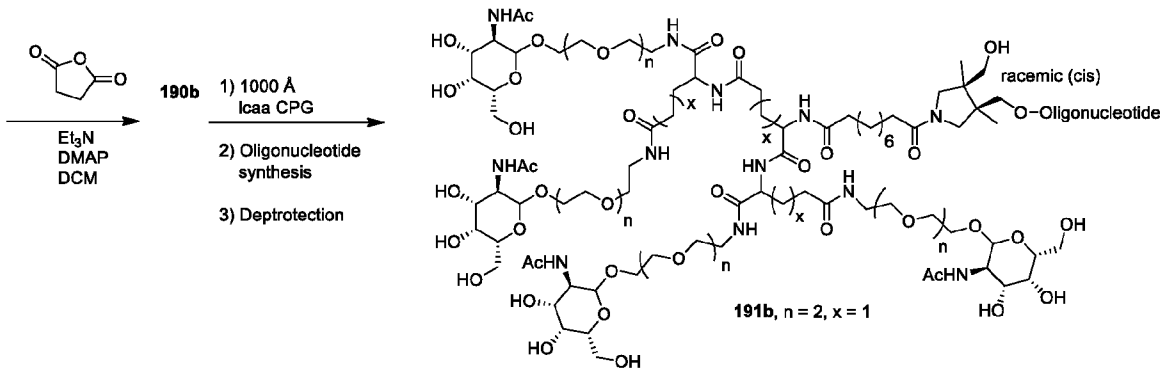


**Scheme 37b.**




**Scheme 38b.**






**Step 1. Preparation of 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol 86b**

To a solution of 2-(2-(2-chloroethoxy)ethoxy)ethan-1-ol (13 g, 77 mmol) in water (200 mL) is added sodium azide (10 g, 154 mmol). The reaction was heated to 100°C for 18 hours. The reaction was cooled to room temperature and poured into a 1L separatory funnel and extracted with dichloromethane (3 x 200 mL). The combine dichloromethane extracts were dried on magnesium sulfate, filtered and concentrated to dryness to afford 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol as a colorless oil (11.7 g).

**Step 2. Preparation of compound 87b**

Compound **87a** is prepared from **86b** (4.95g, 28.3mmol) and **6b** (10g, 25.7mmol) using an identical procedure to that used for compound **84**. Yield: 10g, 77%.

**Step 3. Preparation of compound 88b**

Compound **88a** is prepared from **87b** (10g, 19.8mmol) using an identical procedure to that used for compound **85**. Yield: 7.63g, 65%.

**Step 4. Preparation of compound 89b**

A solution of **88b** (2g, 3.38mmol) and racemic Z-glutamic acid (427mg, 1.52mmol) in $CH_2Cl_2$ (50mL) is treated with HBTU (1.41g, 3.7mmol) and Hünig's base (1.77mL, 10.1mmol). After stirring (18h) the mixture was concentrated and subjected to chromatography to yield **89b** (871mg, 48%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 5. Preparation of compound 90b**

A solution of **89b** (870mg, 0.72mmol) and Pd/C (90mg, 10% - wet support) in EtOAc (10mL) is treated with TFA (84μL, 1.1mmol) and purged with $H_2$. After stirring vigorously (2h) the reaction is purged with $N_2$, filtered through Celite and concentrated. The crude material is used without further processing and yielded **90b** (850mg, quantitative) as a colorless foam. Rf 0.25 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 6. Preparation of compound 91b**

A solution of **90b** (850mg, 0.72mmol) and Z-glutamic acid (91mg, 0.32mmol) in $CH_2Cl_2$ (10mL) is treated with HBTU (300mg, 0.79mmol) and Hünig's base (502μL, 2.9mmol). After stirring (1.5h) the mixture is diluted with $CH_2Cl_2$ and washed with $NaHCO_3$ (Sat. Aq.), dried ($MgSO_4$), filtered and concentrated. The crude material is subjected to chromatography to yield **91b** (590mg, 76%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 7. Preparation of compound 92b**

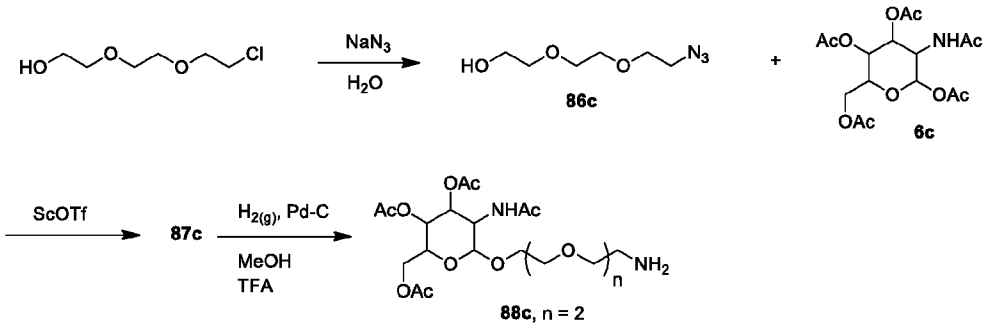
A solution of **91b** (590mg, 0.25mmol) and Pd/C (100mg, 10% - wet support) in $CH_3OH$ (30mL) is treated with TFA (29μL, 0.37mmol) and purged with $H_2$. After stirring (3h) the mixture is purged with $N_2$, then filtered through Celite and concentrated. The crude material is used without further processing and yielded **92b** (600mg, quantitative) as a colorless foam. Rf 0.1 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 8. Preparation of conjugate 191b**

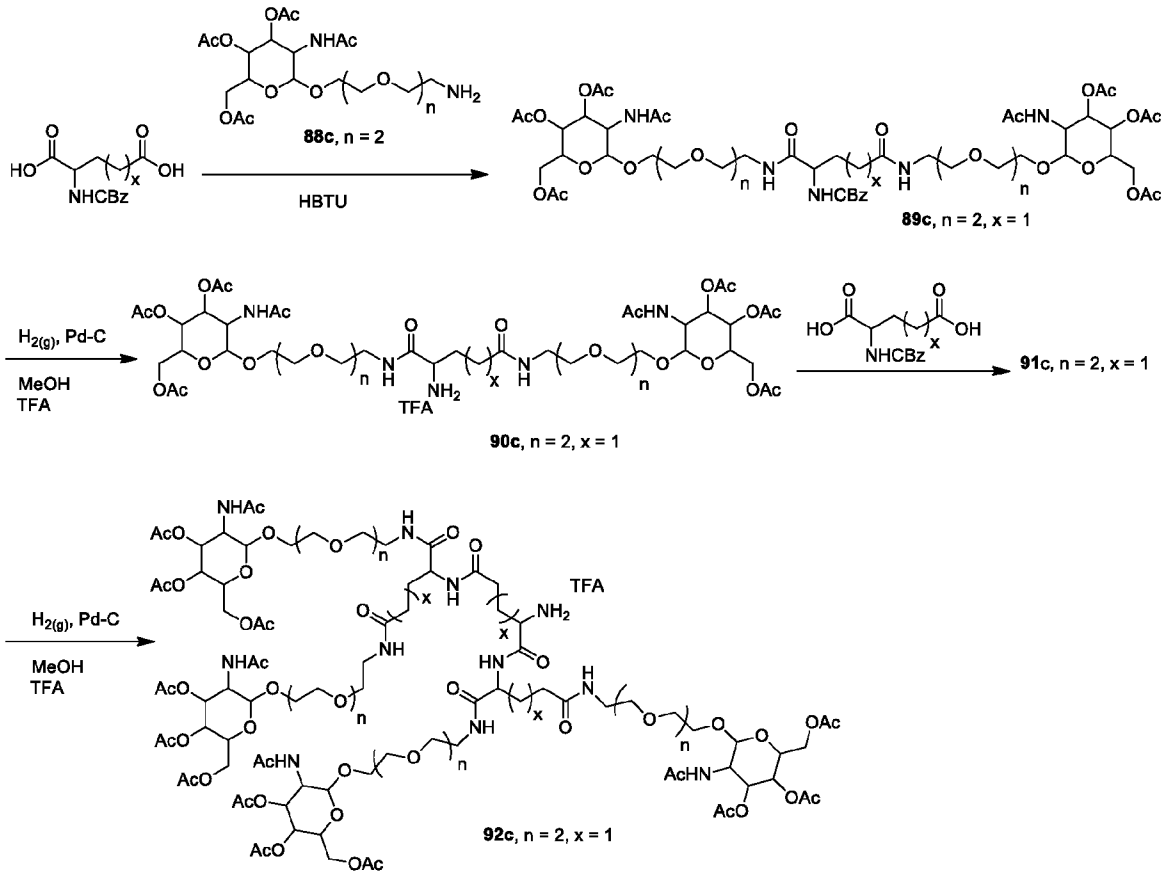
Conjugate **191b** is prepared from compound **128** and compound **92b** using an identical procedure to that used for compound **1**.

## Example 16c. Synthesis of conjugates 191c

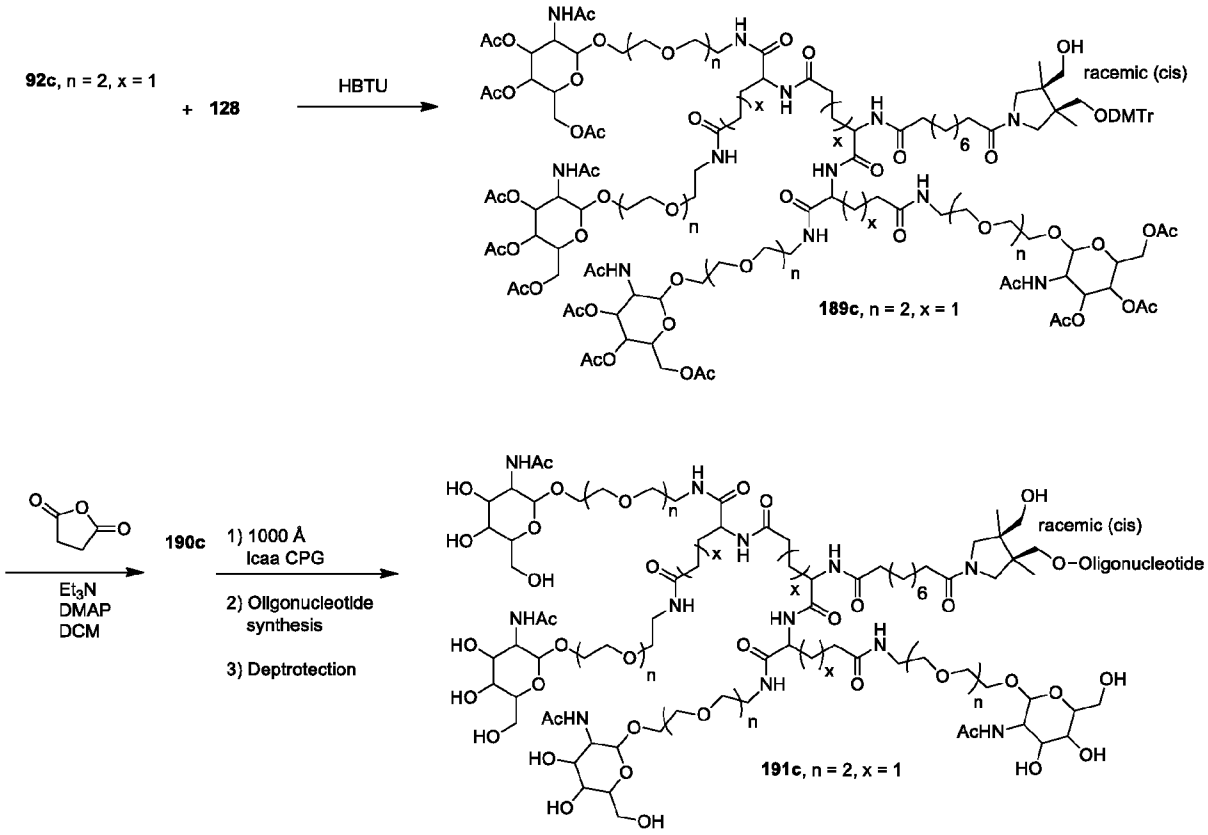
### Scheme 36c




### Scheme 37c.



**Scheme 38c.**




**Step 1. Preparation of 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol 86c**

To a solution of 2-(2-(2-chloroethoxy)ethoxy)ethan-1-ol (13 g, 77 mmol) in water (200 mL) is added sodium azide (10 g, 154 mmol). The reaction was heated to 100°C for 18 hours. The reaction was cooled to room temperature and poured into a 1L separatory funnel and extracted with dichloromethane (3 x 200 mL). The combine dichloromethane extracts were dried on magnesium sulfate, filtered and concentrated to dryness to afford 2-(2-(2-azidoethoxy)ethoxy)ethan-1-ol as a colorless oil (11.7 g).

**Step 2. Preparation of compound 87c**

Compound **87c** is prepared from **86c** (4.95g, 28.3mmol) and **6c** (10g, 25.7mmol) using an identical procedure to that used for compound **84**. Yield: 10g, 77%.

**Step 3. Preparation of compound 88c**

Compound **88c** is prepared from **87c** (10g, 19.8mmol) using an identical procedure to that used for compound **85**. Yield: 7.63g, 65%.

**Step 4. Preparation of compound 89c**

A solution of **88c** (2g, 3.38mmol) and racemic Z-glutamic acid (427mg, 1.52mmol) in $CH_2Cl_2$ (50mL) is treated with HBTU (1.41g, 3.7mmol) and Hünig's base (1.77mL, 10.1mmol). After stirring (18h) the mixture was concentrated and subjected to chromatography to yield **89c** (871mg, 48%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 5. Preparation of compound 90c**

A solution of **89c** (870mg, 0.72mmol) and Pd/C (90mg, 10% - wet support) in EtOAc (10mL) is treated with TFA (84µL, 1.1mmol) and purged with $H_2$. After stirring vigorously (2h) the reaction is purged with $N_2$, filtered through Celite and concentrated. The crude material is used without further processing and yielded **90c** (850mg, quantitative) as a colorless foam. Rf 0.25 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 6. Preparation of compound 91c**

A solution of **90c** (850mg, 0.72mmol) and Z-glutamic acid (91mg, 0.32mmol) in $CH_2Cl_2$ (10mL) is treated with HBTU (300mg, 0.79mmol) and Hünig's base (502µL, 2.9mmol). After stirring (1.5h) the mixture is diluted with $CH_2Cl_2$ and washed with $NaHCO_3$ (Sat. Aq.), dried ($MgSO_4$), filtered and concentrated. The crude material is subjected to chromatography to yield **91c** (590mg, 76%) as a colorless foam. Rf 0.5 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 7. Preparation of compound 92c**

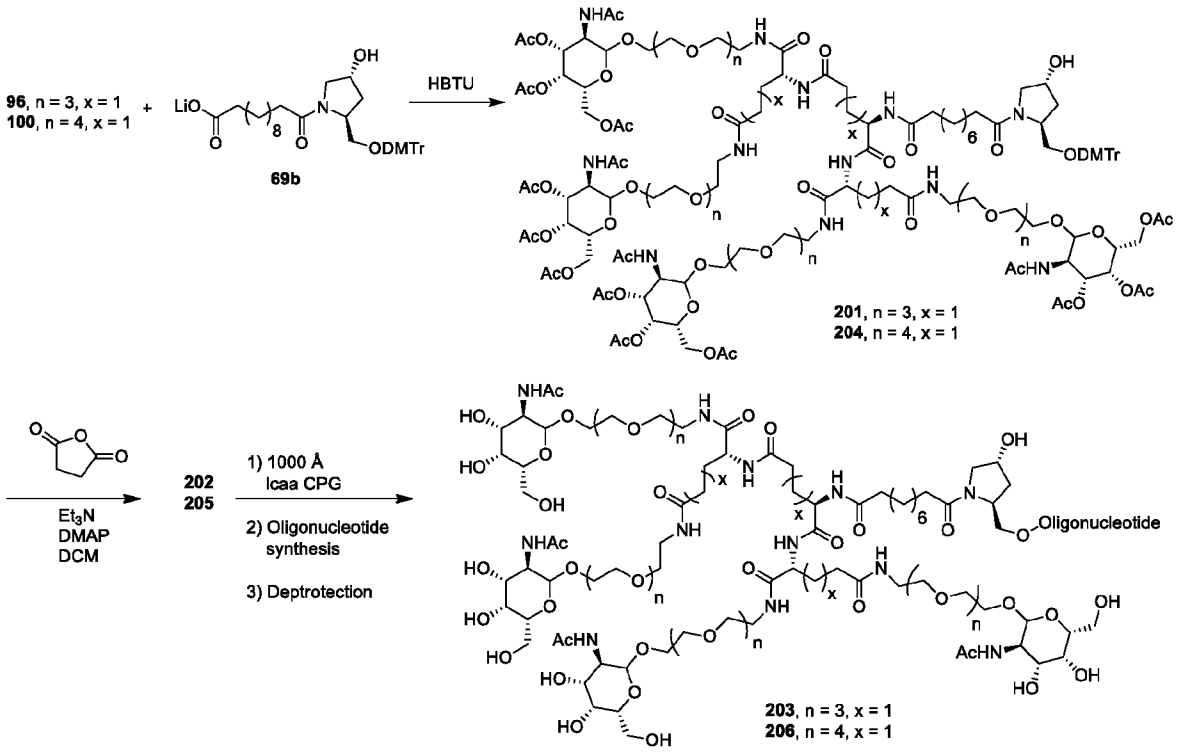
A solution of **91c** (590mg, 0.25mmol) and Pd/C (100mg, 10% - wet support) in $CH_3OH$ (30mL) is treated with TFA (29µL, 0.37mmol) and purged with $H_2$. After stirring (3h) the mixture is purged with $N_2$, then filtered through Celite and concentrated. The crude material is used without further processing and yielded **92c** (600mg, quantitative) as a colorless foam. Rf 0.1 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 8. Preparation of conjugate 191c**

Conjugate **191c** is prepared from compound **128** and compound **92c** using an identical procedure to that used for compound **1**.

## Example 17. Synthesis of conjugates 203 and 206

**Scheme 39.**




### Step 1. Preparation of compound 69b

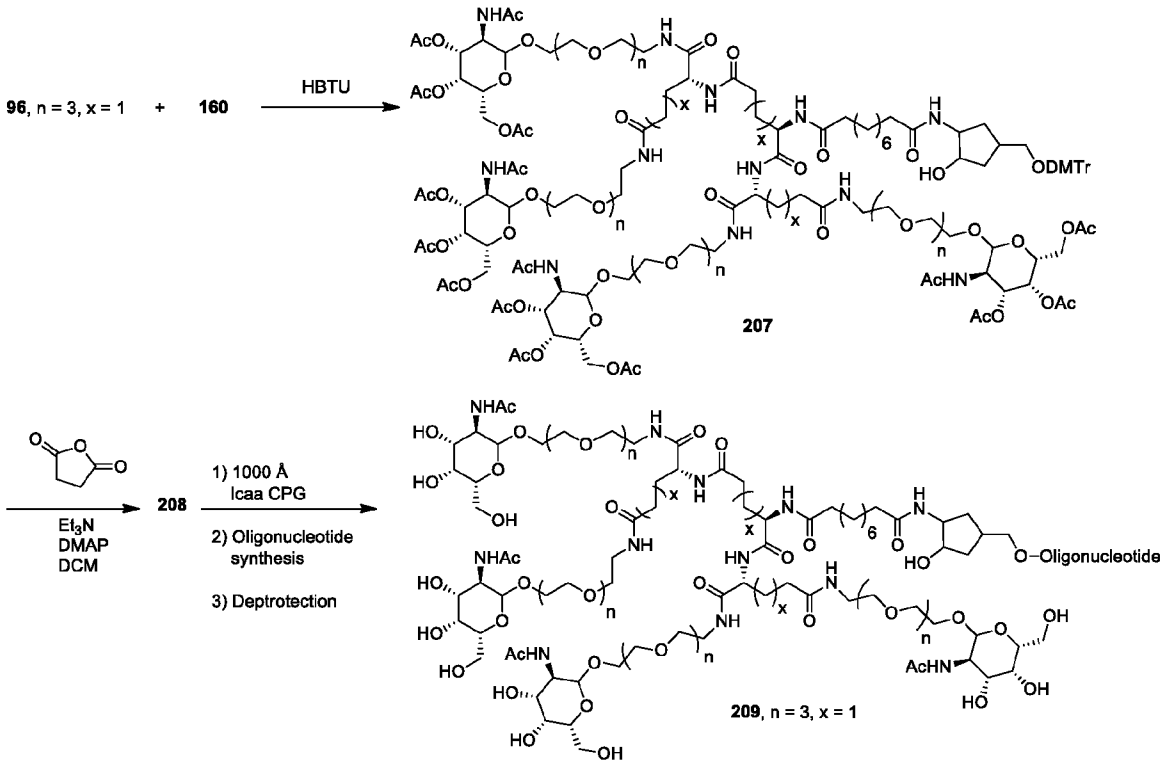
Compound **69b** was prepared from (2S,4R)-4-Hydroxypyrrolidine-2-carboxylic acid using an identical procedure to that used for compound **69**.

### Step 2. Preparation of conjugates 203 and 206

Conjugates **203** and **206** were prepared from compound **96** and **100** using an identical procedure to that used for compound **1**.

## Example 18. Synthesis of conjugate 207

**Scheme 40.**

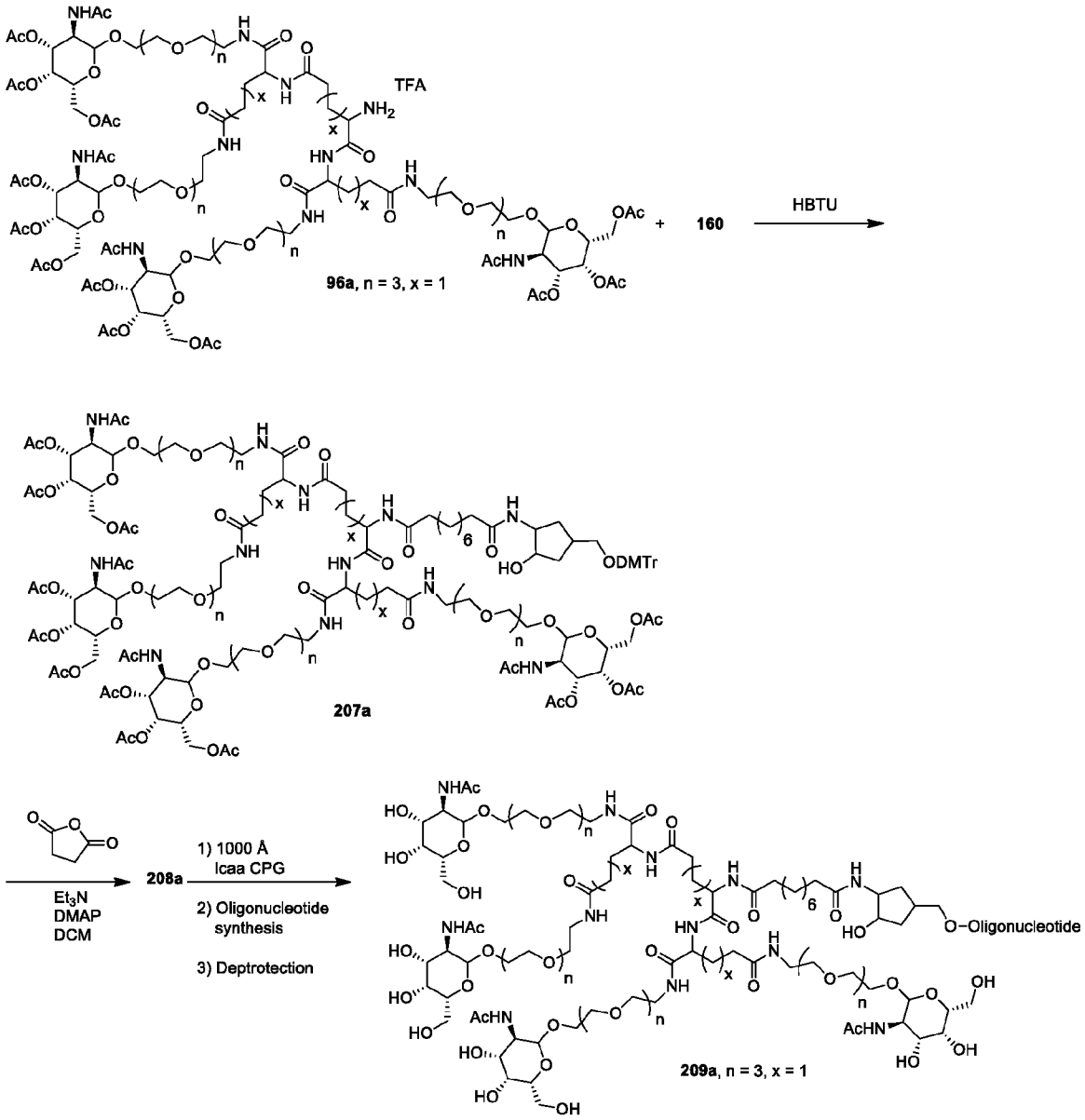



### Step 1. Preparation of conjugate 209

Conjugate **209** was prepared from compound **96** and **160** using an identical procedure to that used for compound **1**.

## Example 18a. Synthesis of conjugate 209a

**Scheme 40a.**

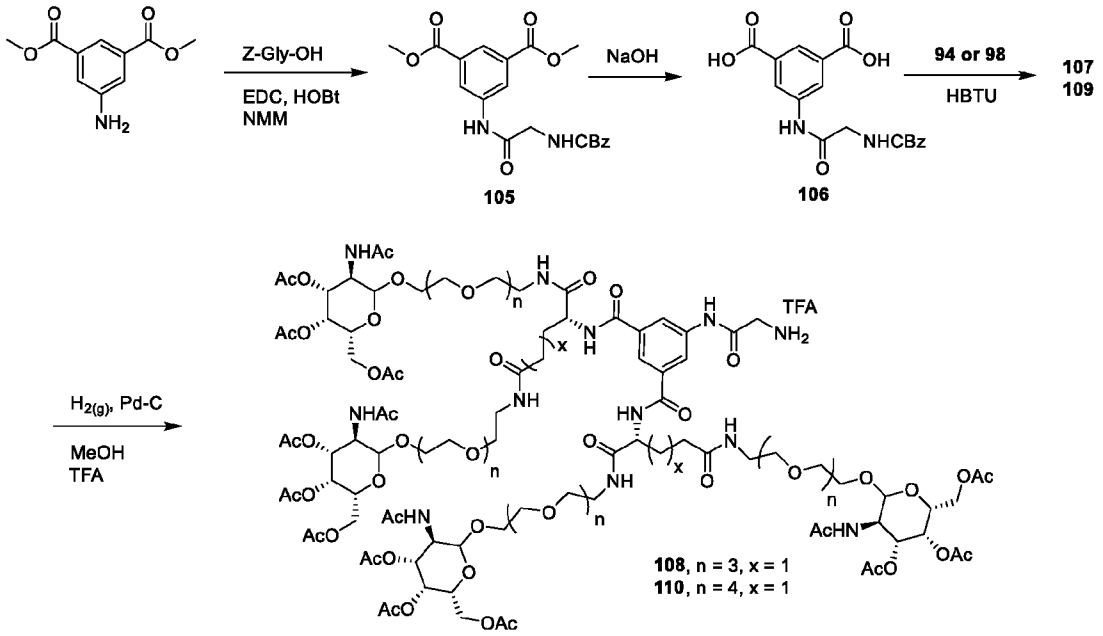



**Step 1. Preparation of conjugate 209a**

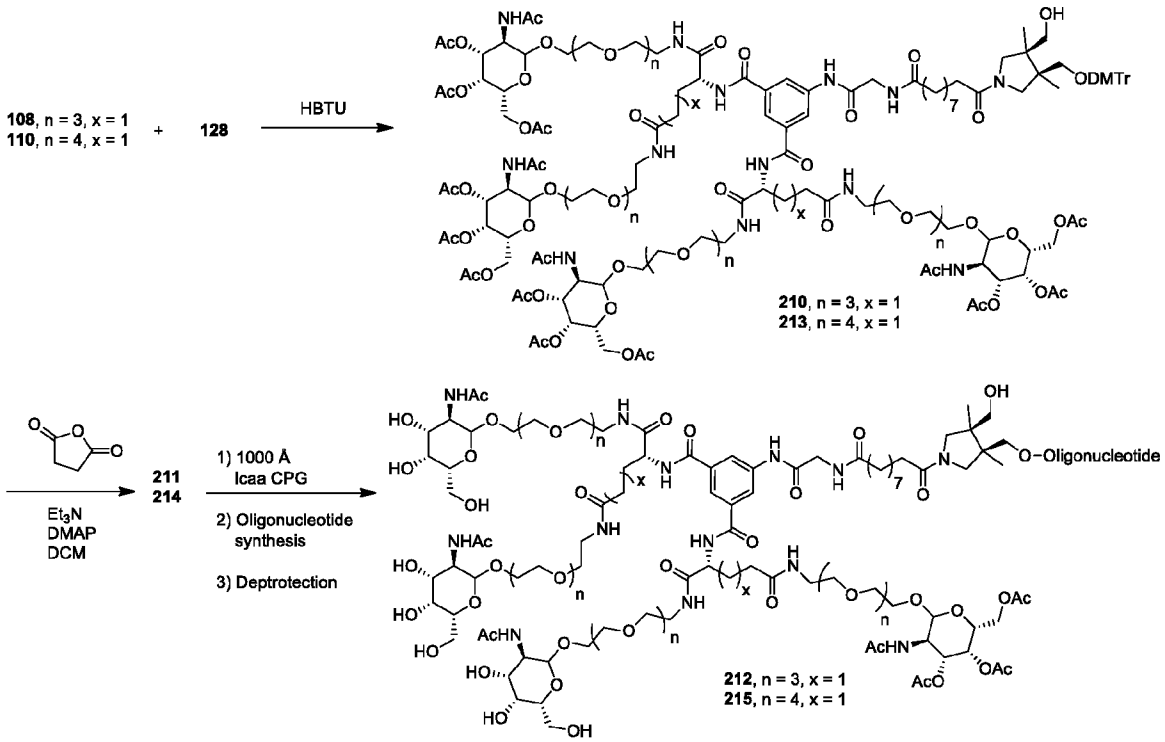
Conjugate **209a** is prepared from compound **96a** and **160** using an identical procedure to that used for compound **1**.

## Example 19. Synthesis of conjugates 212 and 215

**Scheme 41.**




**Scheme 42.**




**Step 1. Preparation of Dimethyl 5-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)acetamido)isophthalate 105**

A solution of dimethyl 5-aminoisophthalate (5 g, 24 mmol), Z-Gly-OH (5 g, 24 mmol), EDC (5 g, 26.3 mmol), HOBt (3.6 g, 26.3 mmol), NMM (2.9 mL, 26.3 mmol) in DMF (50 mL) was stirred overnight at room temperature. Upon completion, the reaction mixture was diluted with ethyl acetate (250 mL) and washed with each 1M HCl (2 x 100 mL), saturated sodium bicarbonate (1 x 100 mL) and brine (2 x 100 mL). Dry on magnesium sulfate, filter and concentrate to dryness to afford Dimethyl 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalate as a colorless solid (7.2 g, 79%).

**Step 2. Preparation of 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalic acid 106**

To a solution of methyl 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalate (7.2 g) in methanol (25 mL) and THF (25 mL) was added 1M NaOH (25 mL). The solution was stirred at room temperature for 2 hours then concentrated to remove THF and MeOH. The aqueous solution remaining was diluted with water (75 mL), cooled on an ice water bath and acidified to pH = 1 with 6M HCl. The solid was filtered and washed with water (3 x 100 mL). The solid was freeze dried to afford 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalic acid (6.9 g, quantitative)

**Step 3. Preparation of compound 107**

Compound **107** was prepared from 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalic acid **106** (200 mg, 0.54 mmol) and **94** (1.7 g, 1.3 mmol) using an identical procedure to that used for compound **95**. Yield: 600 mg.

**Step 4. Preparation of compound 108**

Compound **108** was prepared from compound **107** (600 mg) using an identical procedure to that used for compound **96**. Yield: 650 mg, quantitative.

**Step 5. Preparation of compound 109**

Compound **109** was prepared from 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalic acid **106** (180 mg, 0.48 mmol) and **98** (1.5 g, 1.1 mmol) using an identical procedure to that used for compound **99**. Yield: 900 mg.

**Step 6. Preparation of compound 110**

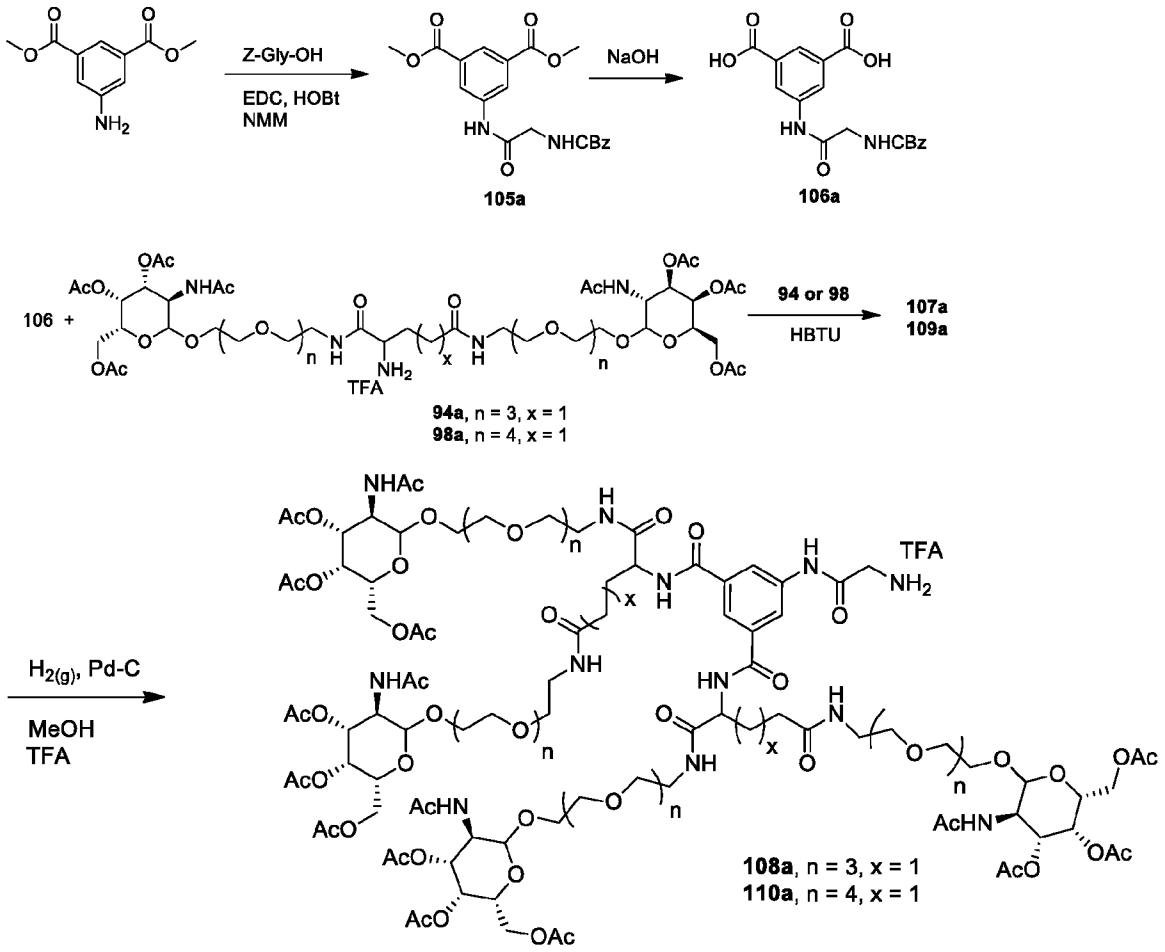
Compound **110** was prepared from compound **109** (900 mg) using an identical procedure to that used for compound **100**. Yield: 920 mg, quantitative.

**Step 7. Preparation of conjugates 212 and 215**

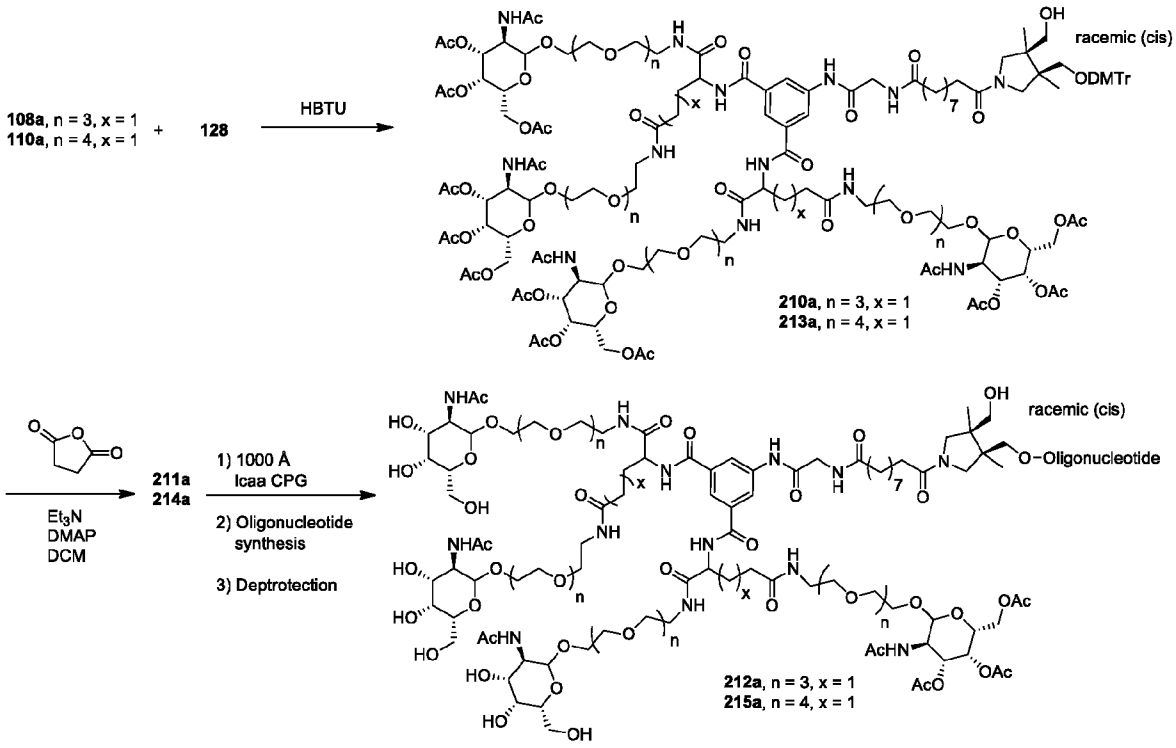
Conjugates **212** and **215** were prepared from compound **128** and **108** or **110** using an identical procedure to that used for compound **1**.

**Example 19a. Synthesis of conjugates 212a and 215a**

**Scheme 41a.**



**Scheme 42a.**




**Step 1. Preparation of Dimethyl 5-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)acetamido)-isophthalate 105a**

A solution of dimethyl 5-aminoisophthalate (5 g, 24 mmol), Z-Gly-OH (5 g, 24 mmol), EDC (5 g, 26.3 mmol), HOBt (3.6 g, 26.3 mmol), NMM (2.9 mL, 26.3 mmol) in DMF (50 mL) is stirred overnight at room temperature. Upon completion, the reaction mixture is diluted with ethyl acetate (250 mL) and washed with each 1M HCl (2 x 100 mL), saturated sodium bicarbonate (1 x 100 mL) and brine (2 x 100 mL). Dry on magnesium sulfate, filter and concentrate to dryness to afford Dimethyl 5-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)acetamido)isophthalate as a colorless solid (7.2 g, 79%).

**Step 2. Preparation of 5-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)acetamido)isophthalic acid 106a**

To a solution of methyl 5-(2-((2-oxo-2-phenyl-1λ$^2$-ethyl)amino)acetamido)isophthalate (7.2 g) in methanol (25 mL) and THF (25 mL) is added 1M NaOH (25 mL). The solution is stirred at room temperature for 2 hours then concentrated to remove THF and MeOH. The aqueous solution remaining is diluted with water (75 mL), cooled on an ice water bath and acidified to pH = 1 with 6M HCl. The solid is filtered and washed with water (3 x 100 mL). The

solid is freeze dried to afford 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)-isophthalic acid (6.9 g, quantitative) .

**Step 3. Preparation of compound 107a**

Compound **107a** is prepared from 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalic acid **106a** (200 mg, 0.54 mmol) and **94a** (1.7 g, 1.3 mmol) using an identical procedure to that used for compound **95**. Yield: 600 mg.

**Step 4. Preparation of compound 108a**

Compound **108a** is prepared from compound **107a** (600 mg) using an identical procedure to that used for compound **96a**. Yield: 650 mg, quantitative.

**Step 5. Preparation of compound 109a**

Compound **109a** is prepared from 5-(2-((2-oxo-2-phenyl-1$\lambda^2$-ethyl)amino)acetamido)isophthalic acid **106a** (180 mg, 0.48 mmol) and **9a8** (1.5 g, 1.1 mmol) using an identical procedure to that used for compound **99**. Yield: 900 mg.

**Step 6. Preparation of compound 110a**

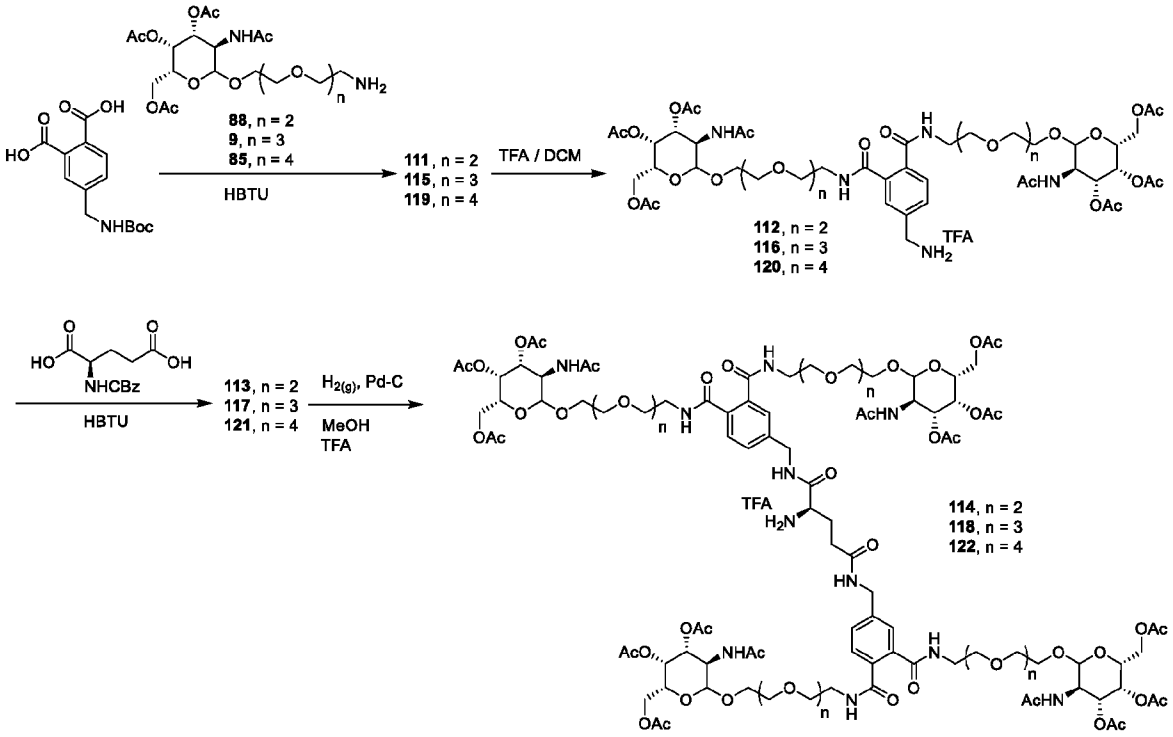
Compound **110a** is prepared from compound **109** (900 mg) using an identical procedure to that used for compound **100**. Yield: 920 mg, quantitative.

**Step 7. Preparation of conjugates 212a and 215a**

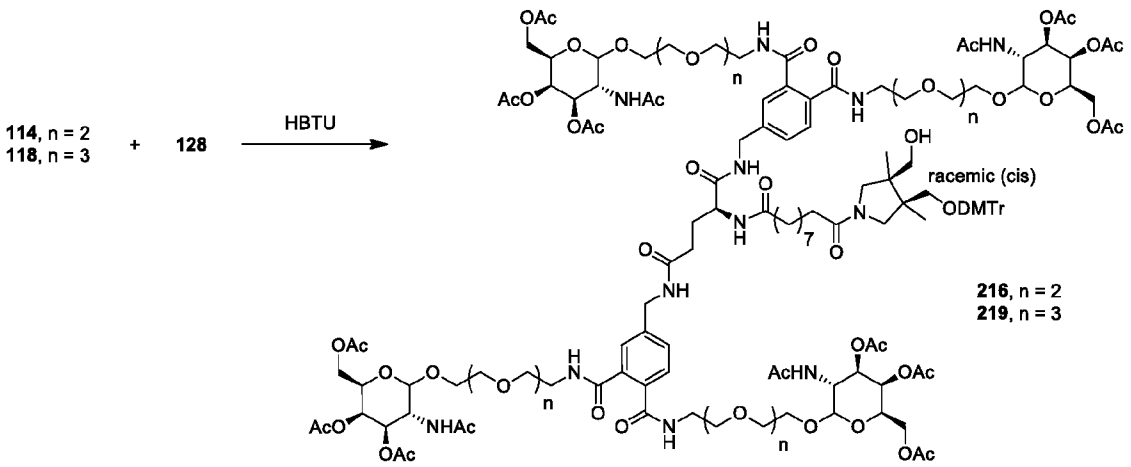
Conjugates **212a** and **21a5** are prepared from compound **128** and **108a** or **110a** using an identical procedure to that used for compound **1**.

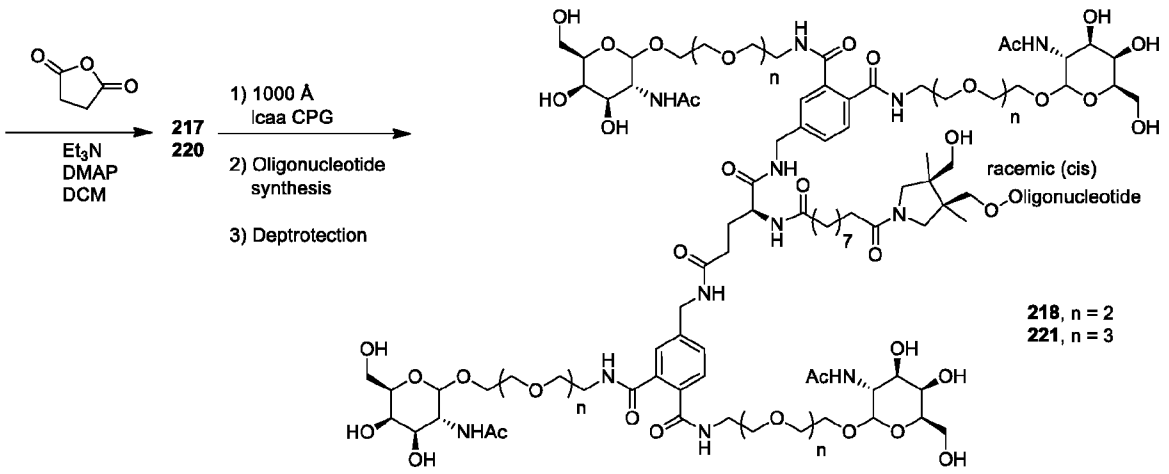
## Example 20. Synthesis of conjugates 218 and 221

**Scheme 43.**




**Scheme 44.**






**Step 1. Preparation of compound 111**

Compound **111** was prepared from 4-(((tert-butoxycarbonyl)amino)methyl)phthalic acid (1.13g, 3.84mmol) and **88** (5g, 8.44mmol) using an identical procedure to that used for compound **89**. Yield: 2.21g, 49%.

**Step 2. Preparation of compound 112**

A solution of **111** (2.21g, 1.87mmol) in $CH_2Cl_2$ (40mL) was slowly treated with TFA (5mL). After stirring (2h) the mixture was concentrated and subjected to chromatography to yield **112** (1.08g, 47%) as a colorless foam. Rf 0.1 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 3. Preparation of compound 113**

Compound **113** was prepared from compound **112** (1.08g, 0.88mmol) and (2-oxo-2-phenyl-1$\lambda^2$-ethyl)-D-glutamic acid (112mg, 0.39mmol) using an identical procedure to that used for compound **91**. Yield: 600mg, 62%.

**Step 4. Preparation of compound 114**

Compound **114** was prepared from compound **113** using an identical procedure to that used for compound **92**.

**Step 5. Preparation of compound 115**

Compound **115** was prepared from 4-(((tert-butoxycarbonyl)amino)methyl)phthalic acid (3.94g, 13.3mmol) and **9** (18.2g, 29.4mmol) using an identical procedure to that used for compound **93**. Yield: 9.02g, 53%.

**Step 6. Preparation of compound 116**

Compound **116** was prepared from compound **115** (8g, 6.3mmol) using an identical procedure to that used for compound **112**. Yield: 3.23g, 39%.

**Step 7. Preparation of compound 117**

Compound **117** was prepared from compound **116** (3.23g, 2.45mmol) and (2-oxo-2-phenyl-1$\lambda^2$-ethyl)-D-glutamic acid (192mg, 1.1mmol) using an identical procedure to that used for compound **95**. Yield: 2.22g, 34%.

**Step 8. Preparation of compound 118**

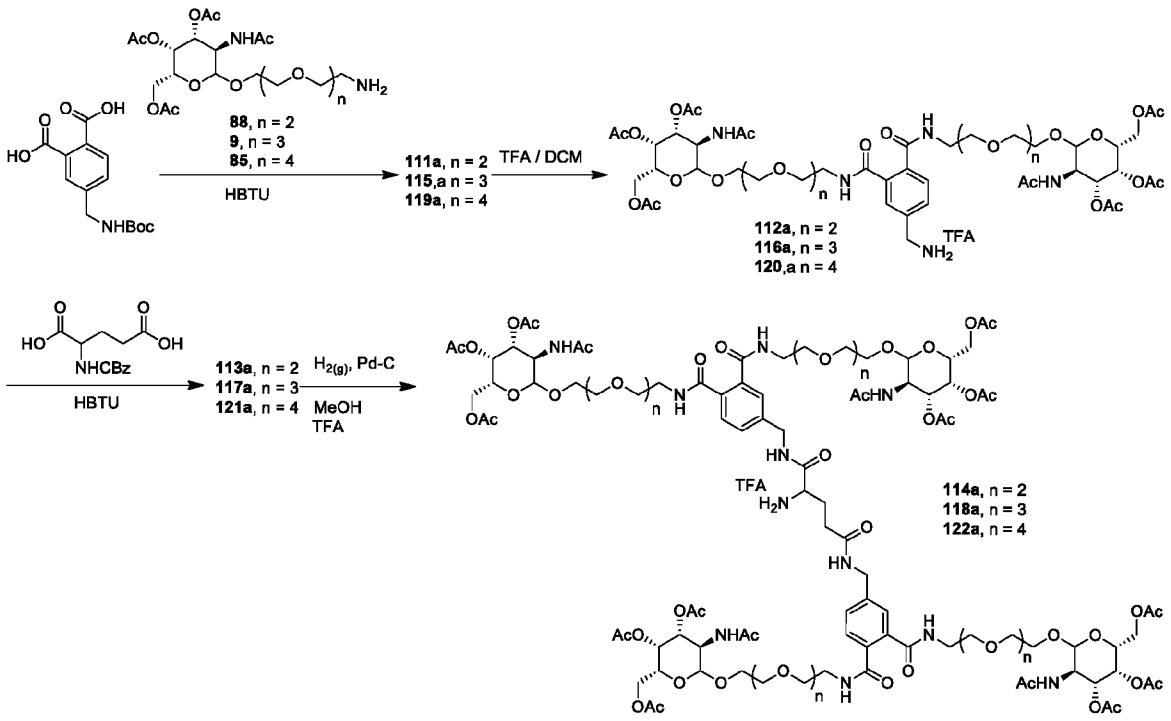
Compound **118** was prepared from compound **117** (2.22g, 0.84mmol) using an identical procedure to that used for compound **96**. Yield: 2.02g, 91%.

**Step 9. Preparation of conjugates 218 and 221**

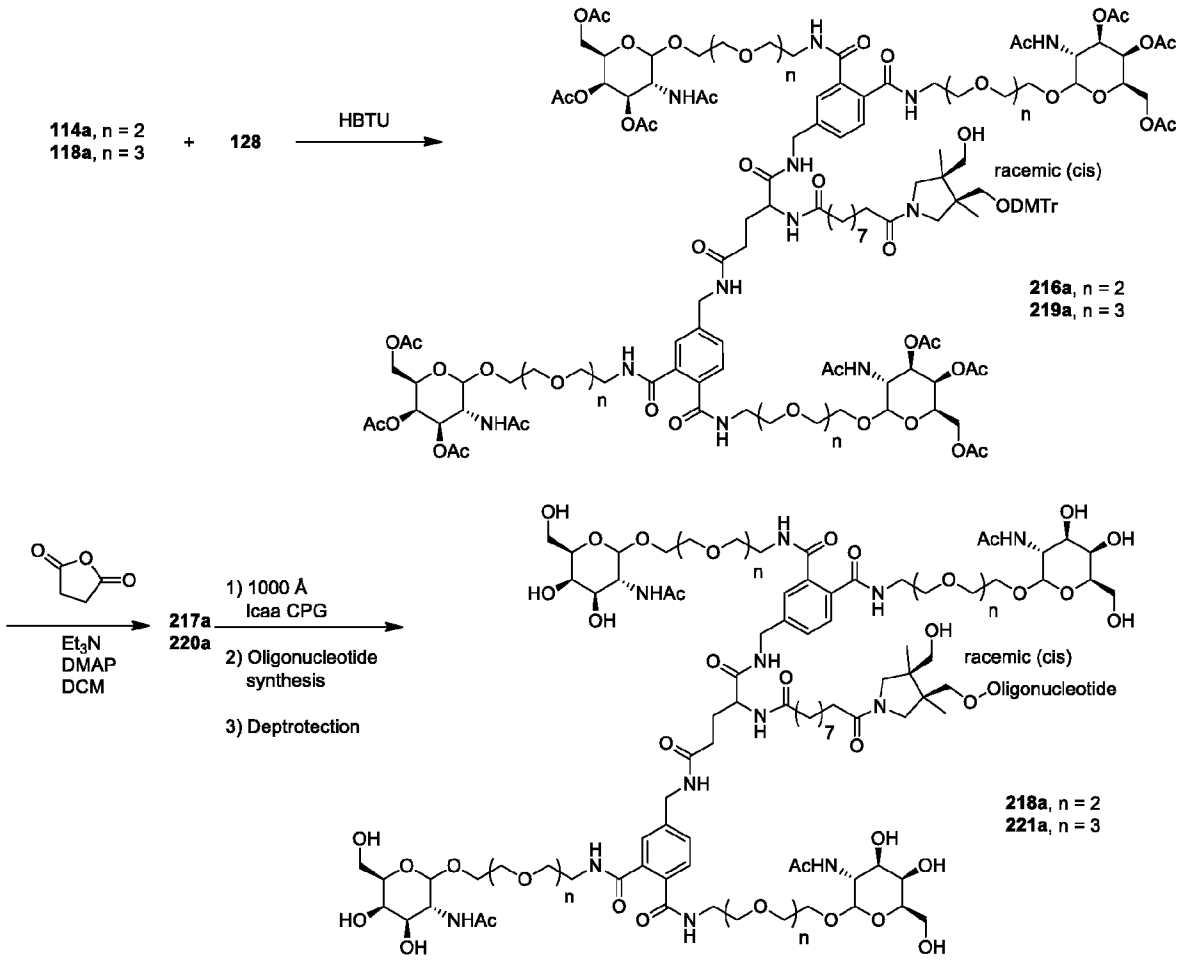
Conjugates **218** and **221** were prepared from compounds **128** and **114** or **118** using an identical procedure to that used for compound **1**.

**Example 20a. Synthesis of conjugates 218a and 221a**

**Scheme 43a.**



**Scheme 44a.**


114a, n = 2
118a, n = 3
+
128
HBTU
racemic (cis)
ODMTr
216a, n = 2
219a, n = 3
Et3N
DMAP
DCM
217a
220a
1) 1000 Å
Icaa CPG
2) Oligonucleotide
synthesis
3) Deptrotection
racemic (cis)
Oligonucleotide
218a, n = 2
221a, n = 3

**Step 1. Preparation of compound 111a**

Compound **111a** is prepared from 4-(((tert-butoxycarbonyl)amino)methyl)phthalic acid (1.13g, 3.84mmol) and **88** (5g, 8.44mmol) using an identical procedure to that used for compound **89**. Yield: 2.21g, 49%.

**Step 2. Preparation of compound 112a**

A solution of **111a** (2.21g, 1.87mmol) in $CH_2Cl_2$ (40mL) is slowly treated with TFA (5mL). After stirring (2h) the mixture is concentrated and subjected to chromatography to yield **112a** (1.08g, 47%) as a colorless foam. Rf 0.1 (10% $CH_3OH$-$CH_2Cl_2$).

**Step 3. Preparation of compound 113a**

Compound **113a** is prepared from compound **112a** (1.08g, 0.88mmol) and (2-oxo-2-phenyl-1$\lambda^2$-ethyl)-D-glutamic acid (112mg, 0.39mmol) using an identical procedure to that used for compound **91**. Yield: 600mg, 62%.

**Step 4. Preparation of compound 114a**

Compound **114a** is prepared from compound **113a** using an identical procedure to that used for compound **92**.

**Step 5. Preparation of compound 115a**

Compound **115a** is prepared from 4-(((tert-butoxycarbonyl)amino)methyl)phthalic acid (3.94g, 13.3mmol) and **9** (18.2g, 29.4mmol) using an identical procedure to that used for compound **93**. Yield: 9.02g, 53%.

**Step 6. Preparation of compound 116a**

Compound **116a** is prepared from compound **115a** (8g, 6.3mmol) using an identical procedure to that used for compound **11a**. Yield: 3.23g, 39%.

**Step 7. Preparation of compound 117a**

Compound **117a** is prepared from compound **116a** (3.23g, 2.45mmol) and (2-oxo-2-phenyl-1$\lambda^2$-ethyl)glutamic acid (192mg, 1.1mmol) using an identical procedure to that used for compound **95**. Yield: 2.22g, 34%.

**Step 8. Preparation of compound 118a**

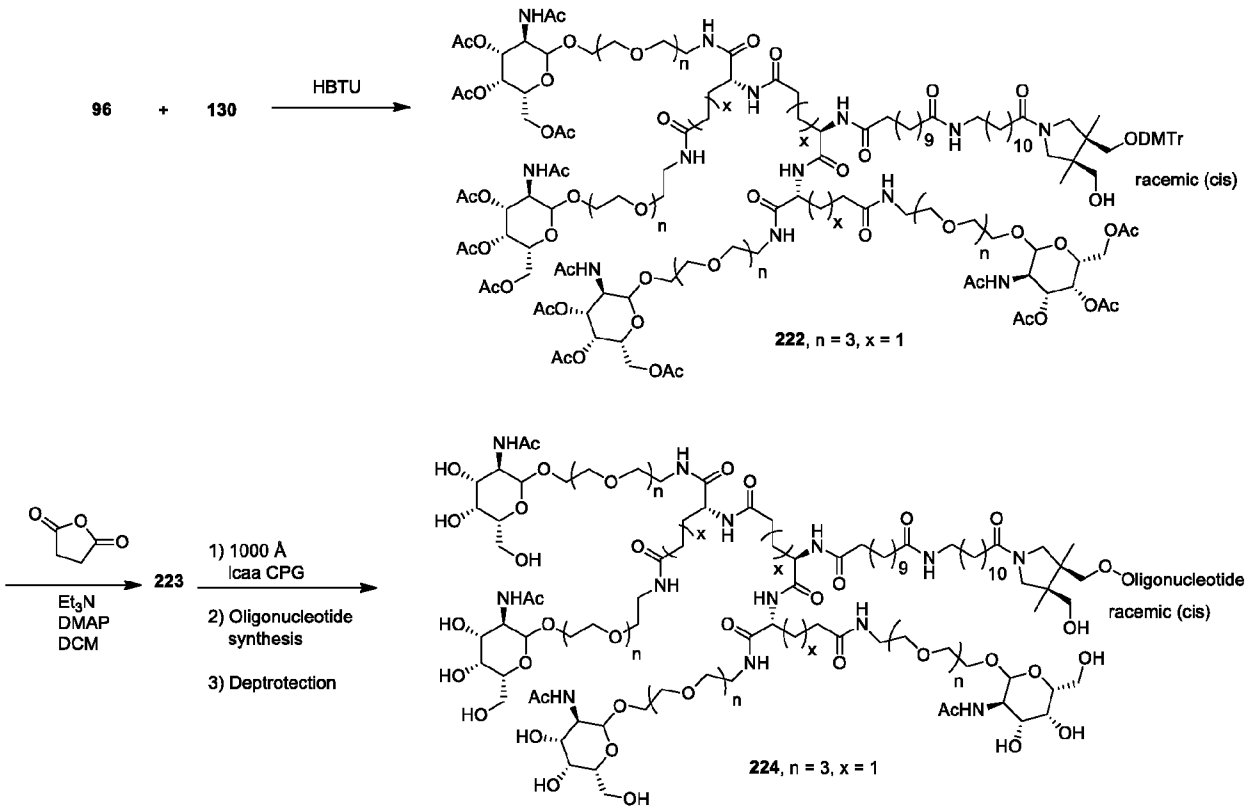
Compound **118a** is prepared from compound **117a** (2.22g, 0.84mmol) using an identical procedure to that used for compound **96**. Yield: 2.02g, 91%.

**Step 9. Preparation of conjugates 21a8 and 221a**

Conjugates **218a** and **22a1** are prepared from compounds **128** and **114a** or **118a** using an identical procedure to that used for compound **1**.

**Example 21. Synthesis of conjugate 224**

**Scheme 45.**

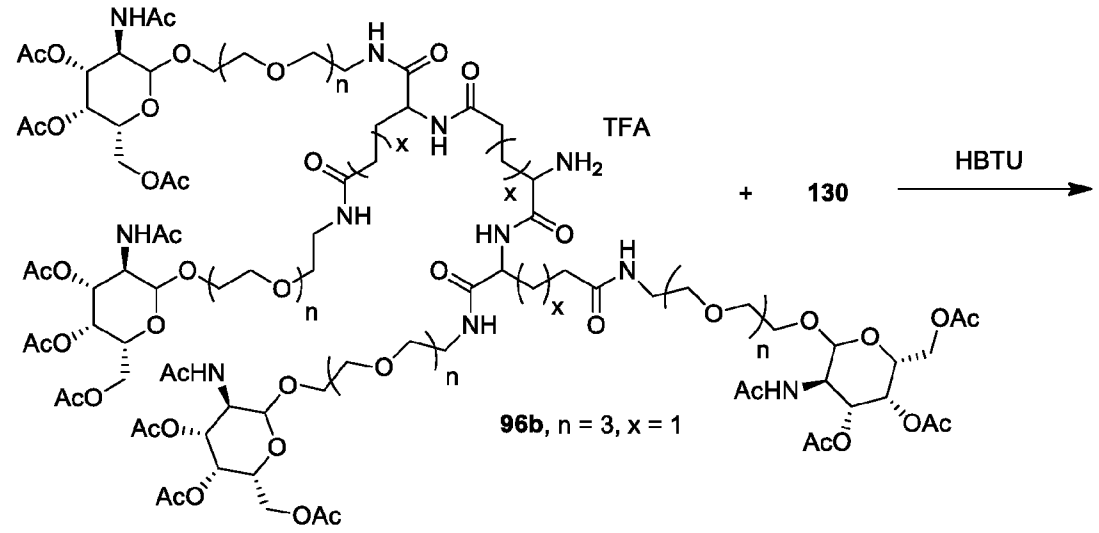


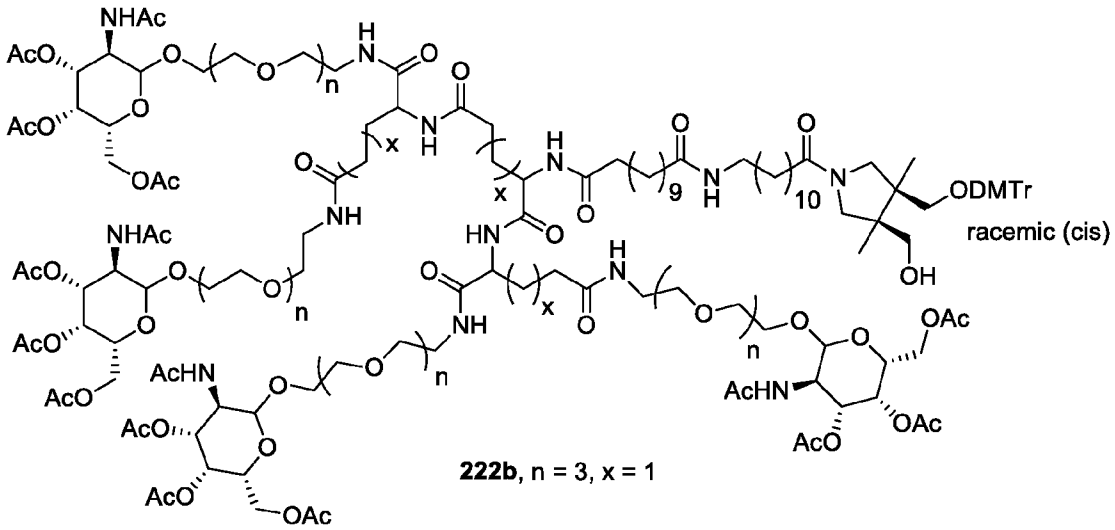

**Step 1. Preparation of compounds 224**

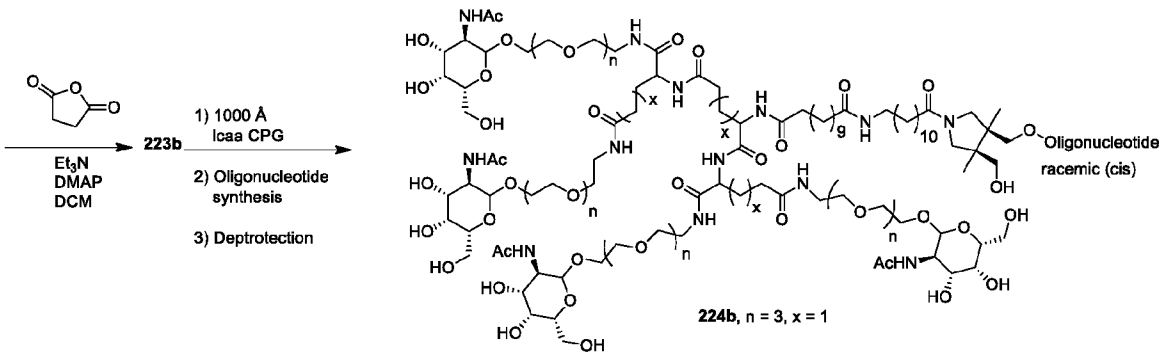
Conjugate **224** was prepared from compounds **96** and **130** using an identical procedure to that used for compound **1**.

## Example 21a. Synthesis of conjugate 224b

**Scheme 45a.**



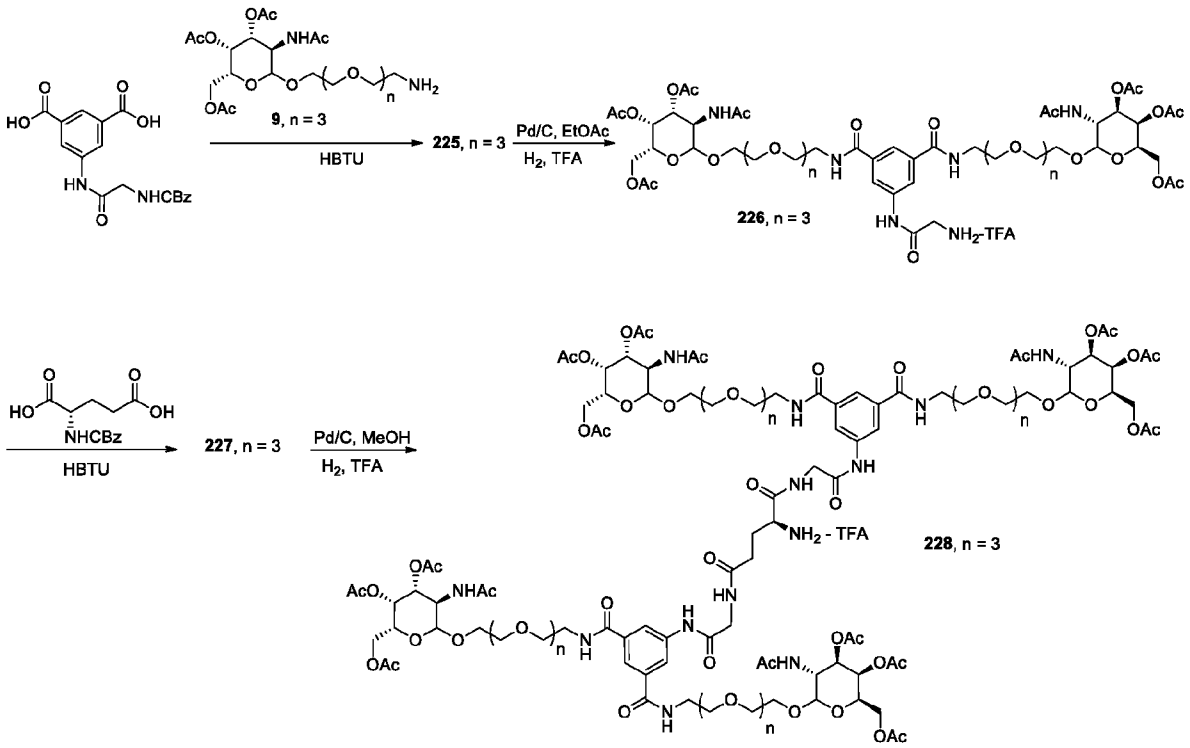



**Step 1. Preparation of compounds 224b**

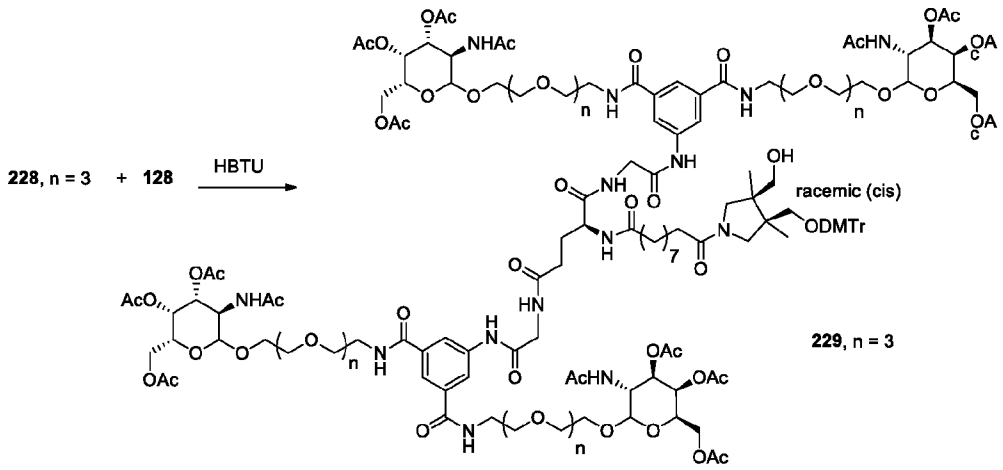
Conjugate **224b** is prepared from compounds **96b** and **130** using an identical procedure to that used for compound **1**.

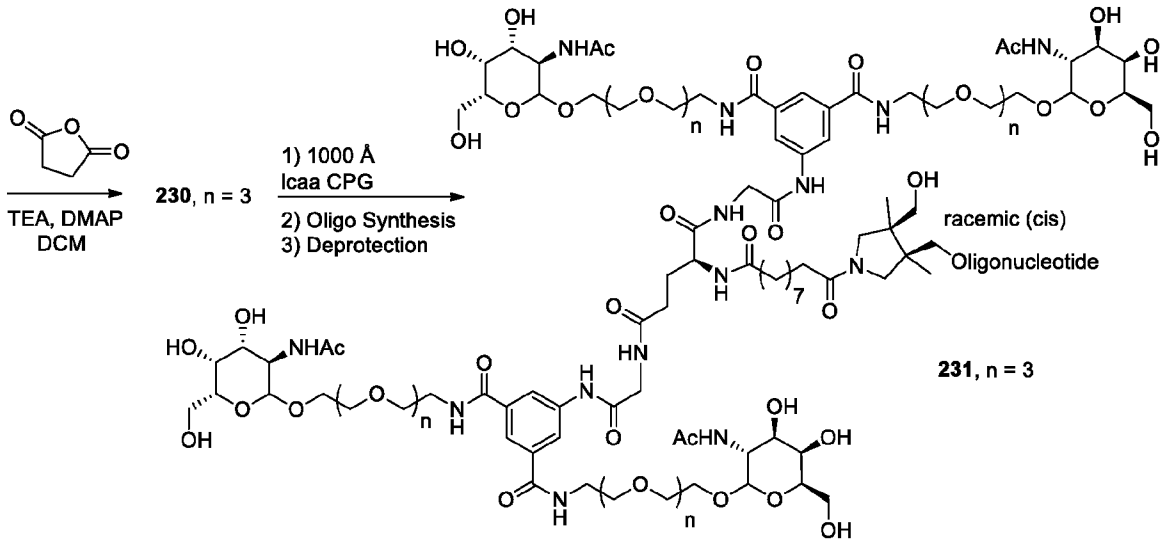
## Example 22 Synthesis of Conjugate 231

**Scheme 46**




**Scheme 47**






**Step 1 Preparation of compound 225**

Compound **225** was prepared from 5-(2-aminoacetamido)isophthalic acid **106** (560mg, 1.5mmol) and **9** (2.24g, 3.6mmol) using an identical procedure to that used for **89**. Yield 1.6g, 80%.

**Step 2 Preparation of compound 226**

Compound **226** was prepared in the same fashion as **14**. Yield 1.22g, 78%.

**Step 3 Preparation of compound 227**

Compound **227** was prepared in the same fashion as **89**, from Z-glutamic acid (108mg, 0.38mmol) and **226** (1.22g, 0.92mmol). Yield 471mg, 45%.

**Step 4 Preparation of compound 228**

Compound **228** was prepared in the same fashion as **14**. Yield 460mg, Quant.

**Step 5 Preparation of compound 229**

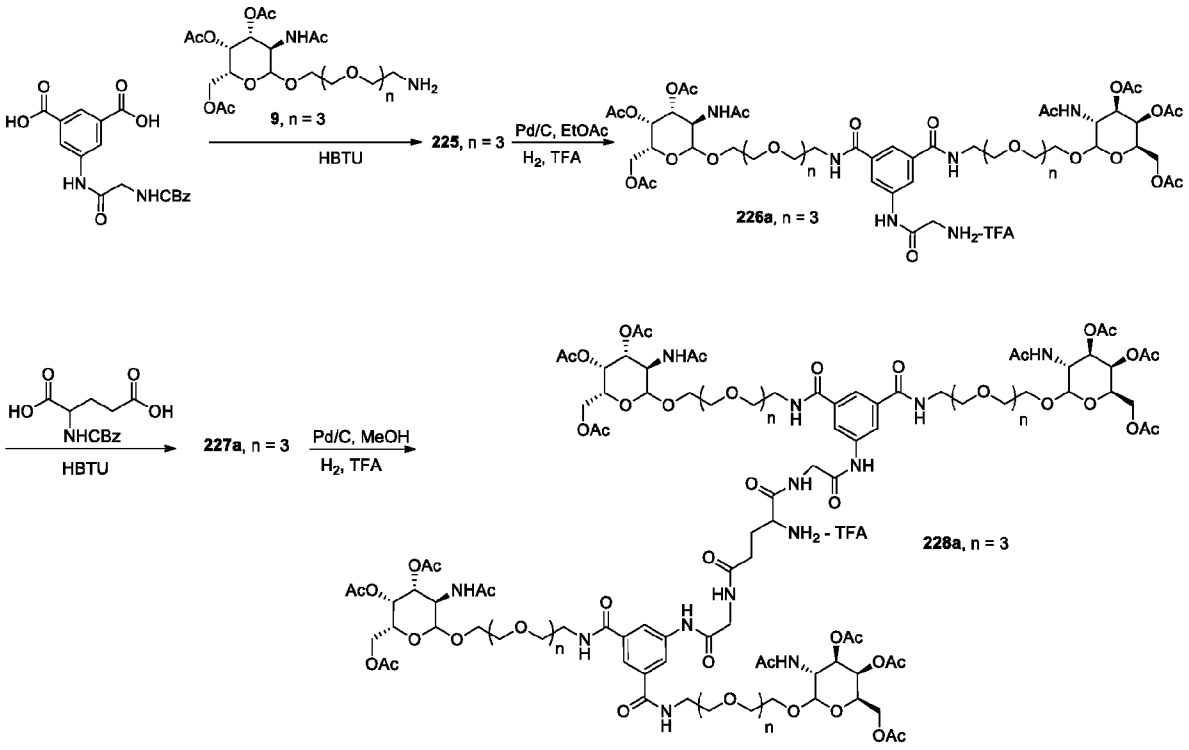
Compound **229** was prepared from **228** (460mg, 0.17mmol) and 128 (125mg, 0.19mmol) in the same fashion as **89**. Yield 365mg, 66%.

**Step 6 Preparation of compound 231**

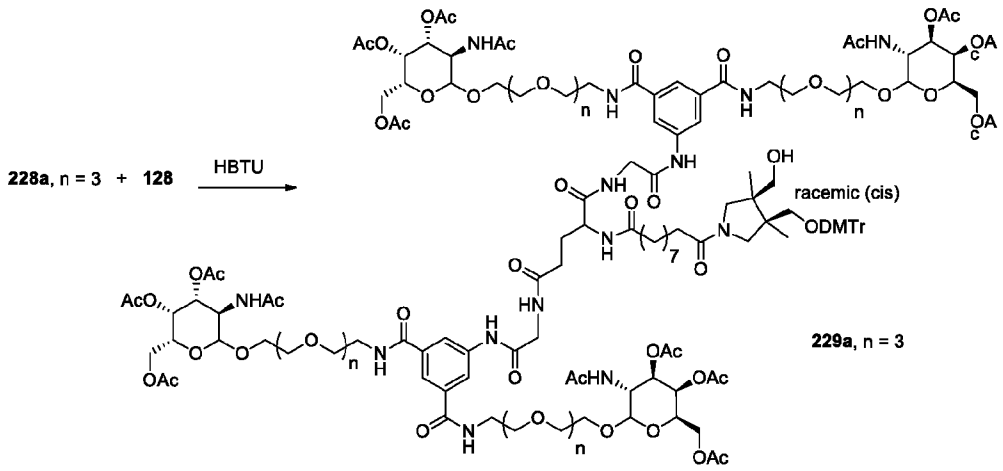
Conjugate **231** was prepared using an identical procedure to that used for compound **1**.

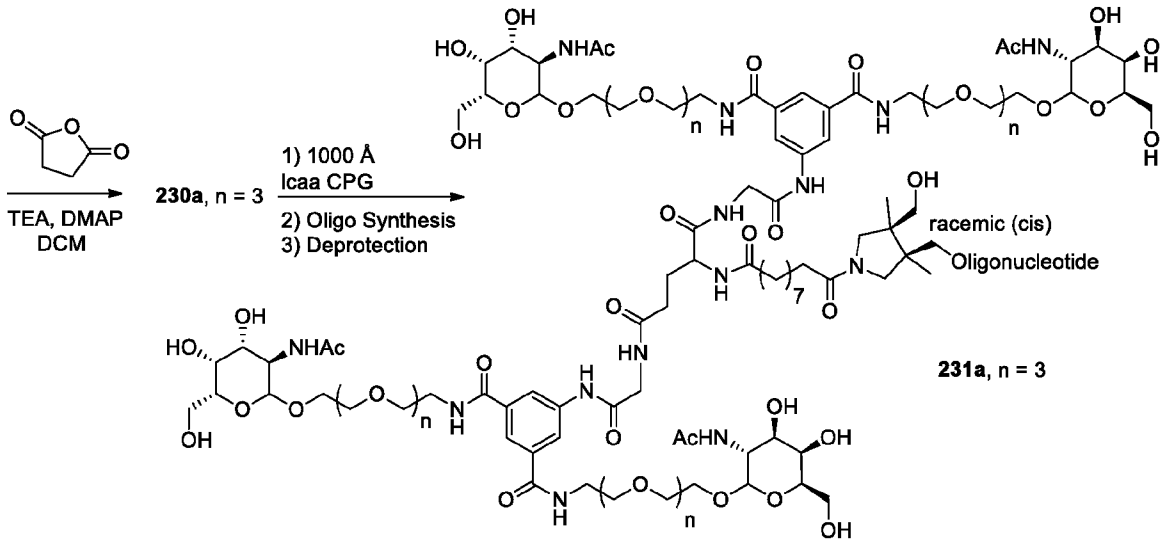
## Example 22a Synthesis of Conjugate 231a

### Scheme 46a




### Scheme 47a






**Step 1 Preparation of compound 225a**

Compound **225a** is prepared from 5-(2-aminoacetamido)isophthalic acid **106** (560mg, 1.5mmol) and **9** (2.24g, 3.6mmol) using an identical procedure to that used for **89**. Yield 1.6g, 80%.

**Step 2 Preparation of compound 226a**

Compound **226a** is prepared in the same fashion as **14**. Yield 1.22g, 78%.

**Step 3 Preparation of compound 227a**

Compound **227a** is prepared in the same fashion as **89**, from Z-glutamic acid (108mg, 0.38mmol) and **226a** (1.22g, 0.92mmol). Yield 471mg, 45%.

**Step 4 Preparation of compound 228a**

Compound **228a** is prepared in the same fashion as **14**. Yield 460mg, Quant.

**Step 5 Preparation of compound 229a**

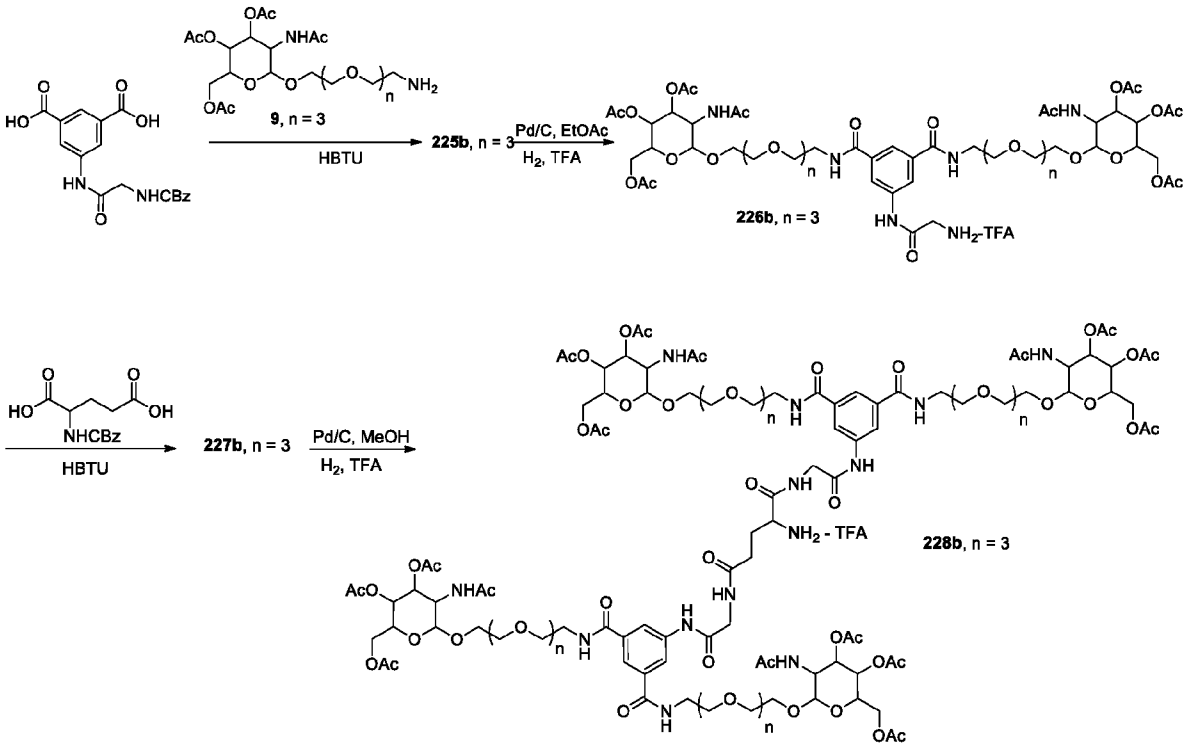
Compound **229a** is prepared from **228a** (460mg, 0.17mmol) and 128 (125mg, 0.19mmol) in the same fashion as **89**. Yield 365mg, 66%.

**Step 6 Preparation of compound 231a**

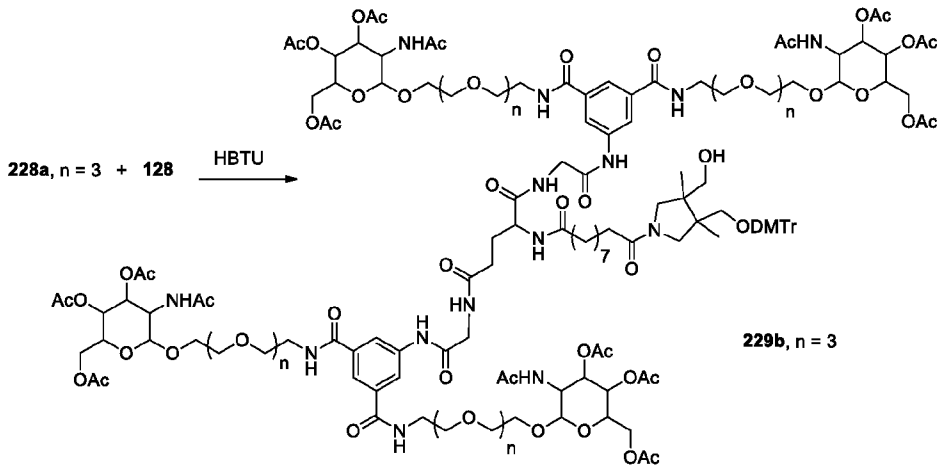
Conjugate **231a** is prepared using an identical procedure to that used for compound **1**.

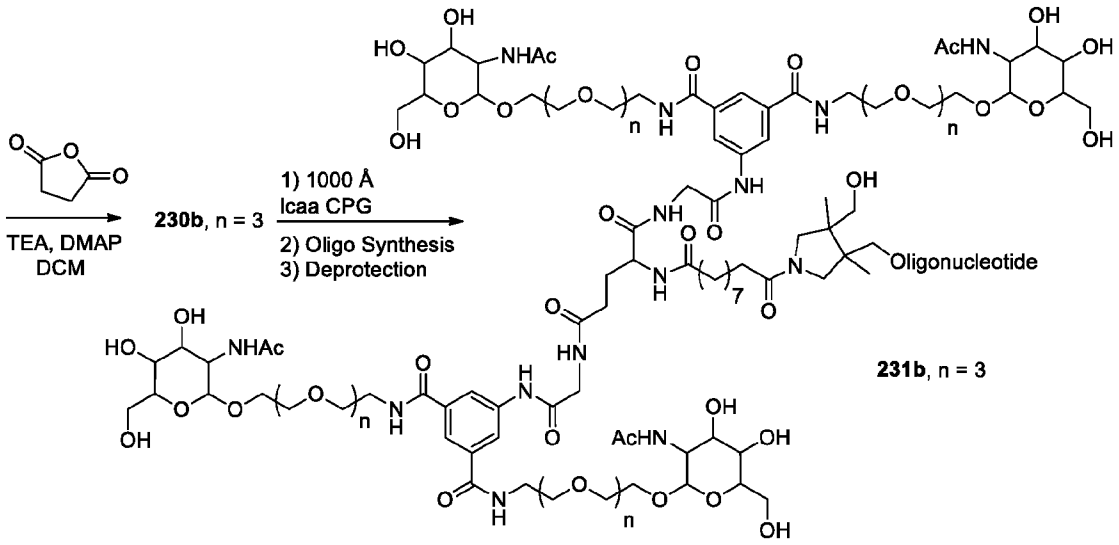
## Example 22b Synthesis of Conjugate 231b

### Scheme 46b




### Scheme 47b






**Step 1 Preparation of compound 225b**

Compound **225b** is prepared from 5-(2-aminoacetamido)isophthalic acid **106** (560mg, 1.5mmol) and **9** (2.24g, 3.6mmol) using an identical procedure to that used for **89**. Yield 1.6g, 80%.

**Step 2 Preparation of compound 226b**

Compound **226b** is prepared in the same fashion as **14**. Yield 1.22g, 78%.

**Step 3 Preparation of compound 227b**

Compound **227b** is prepared in the same fashion as **89**, from Z-glutamic acid (108mg, 0.38mmol) and **226b** (1.22g, 0.92mmol). Yield 471mg, 45%.

**Step 4 Preparation of compound 228b**

Compound **228b** is prepared in the same fashion as **14**. Yield 460mg, Quant.

**Step 5 Preparation of compound 229b**

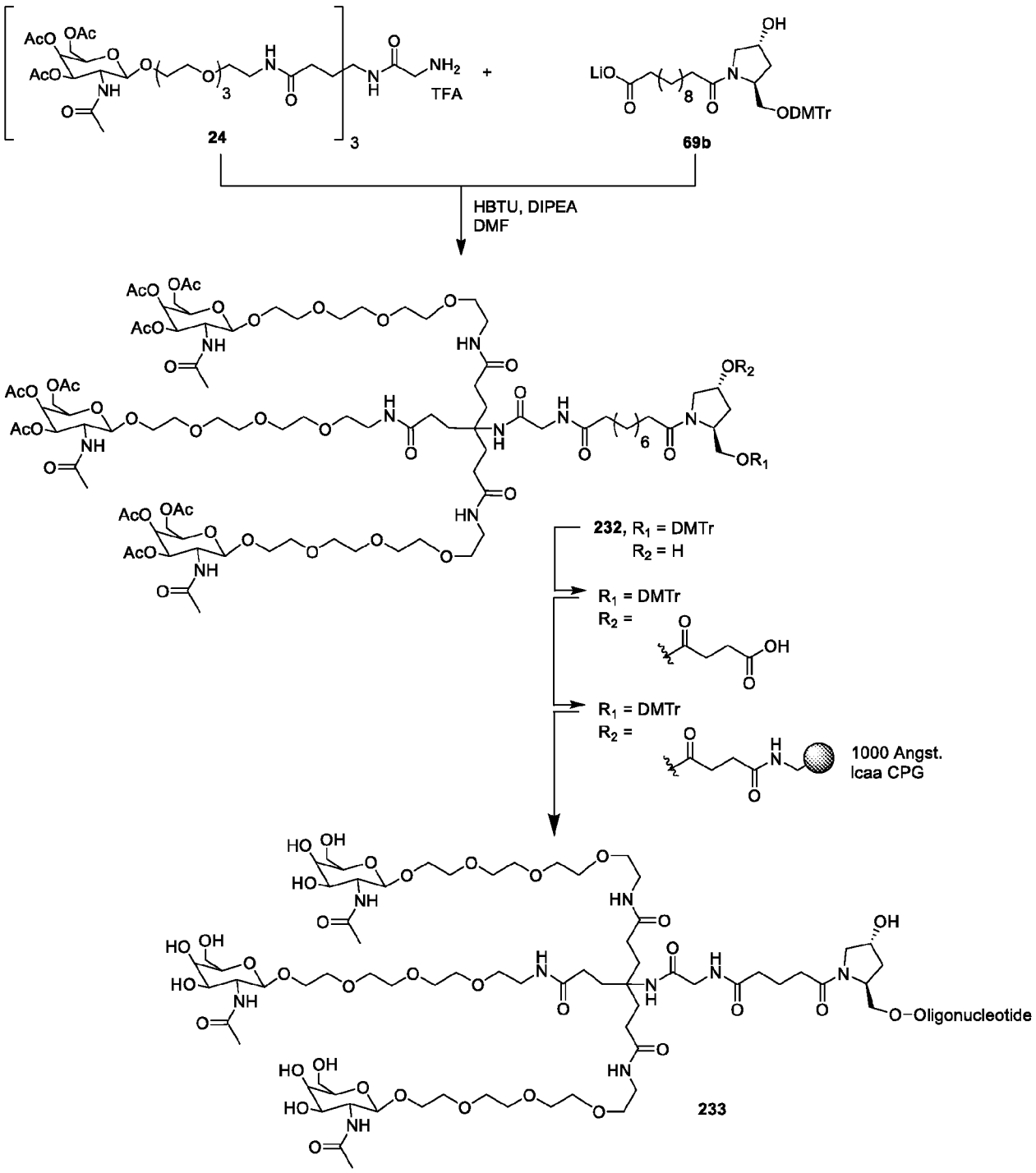
Compound **229b** is prepared from **228b** (460mg, 0.17mmol) and 128 (125mg, 0.19mmol) in the same fashion as **89**. Yield 365mg, 66%.

**Step 6 Preparation of compound 231b**

Conjugate **231b** is prepared using an identical procedure to that used for compound **1**.

## Example 23. Synthesis of conjugate 233

**Scheme 48**



**Step 1. Preparation of compound 232**

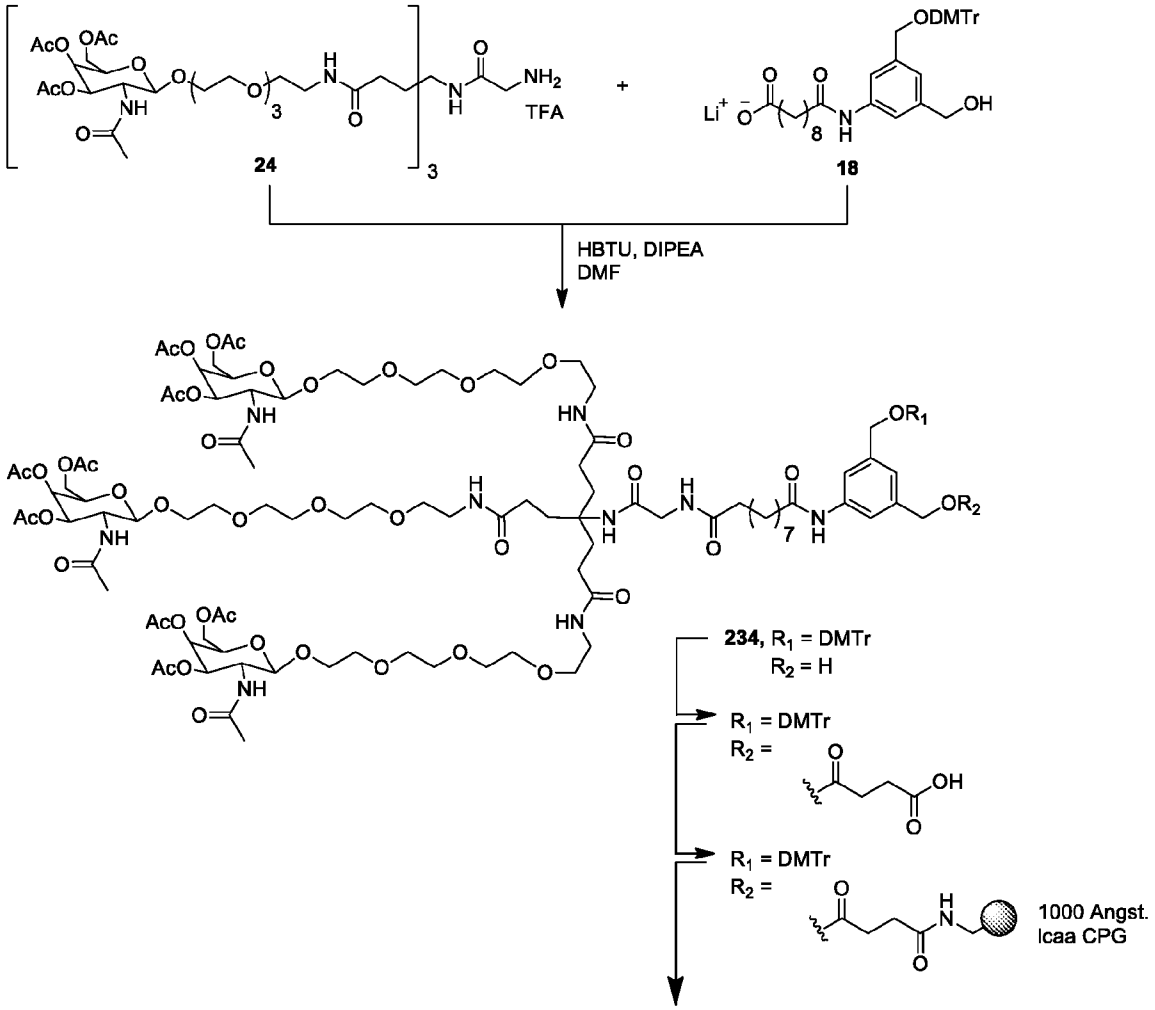
Compound **232** was prepared from compound **24** (650 mg, 0.33 mmol) and compound **69b** (175 mg, 0.33 mmol) using an identical procedure to that used for compound **19**. Yield: 380 mg, 47%.

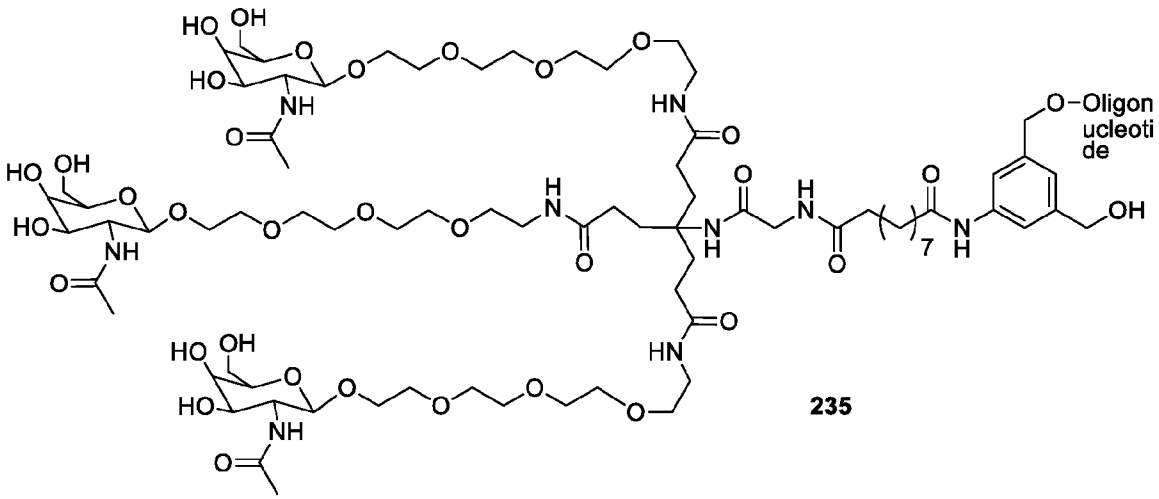
**Step 2. Preparation of compound 233**

Compound **233** was prepared from compound **232** using identical procedures to that used for compound **1**.

## Example 24. Synthesis of conjugate 235

**Scheme 49**






**Step 1. Preparation of compound 234**

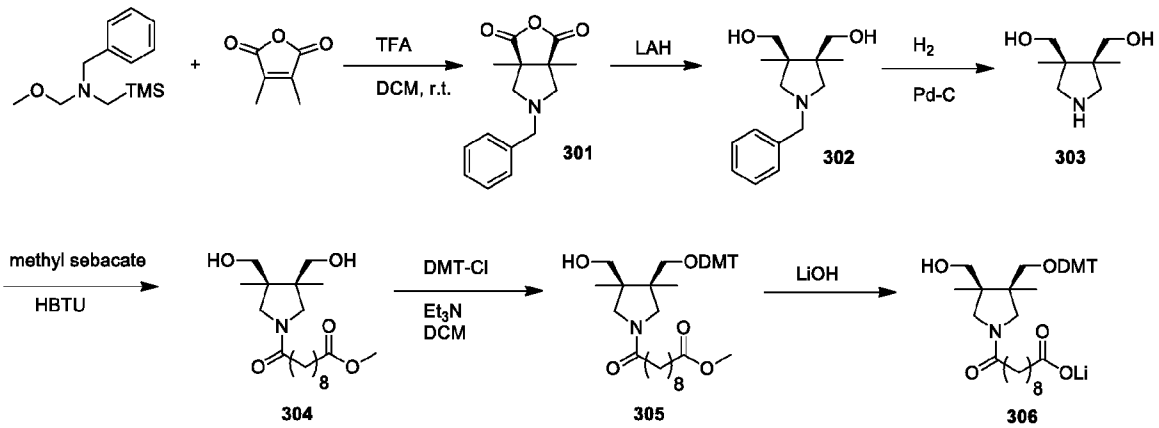
Compound **234** was prepared from compound **24** (1.1 g, 0.55 mmol) and compound **18** (175 mg, 0.33 mmol) using an identical procedure to that used for compound **19**. Yield: 685 mg, 51%.

**Step 2. Preparation of compound 235**

Compound **235** was prepared from compound **234** using identical procedures to that used for compound **1**.

**Example 25. Synthesis of conjugate 320**

**Scheme 50 Preparation of activated linker**



**Step 1. Preparation of Racemic (cis) 5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione 301**

To a cooled solution (0°C) of 3,4-dimethylfuran-2,5-dione (3 g, 24 mmol) and N-benzyl-1-methoxy-N-((trimethylsilyl)methyl)methanamine (7 g, 29.8 mmol) in dichloromethane (75 mL) was slowly added trifluoroacetic acid (75 µL). Stir overnight allowing the solution to slowly warm to room temperature as the ice bath melted. The reaction mixture was concentrated to dryness, dissolved in ethyl acetate (100 mL), washed with saturated sodium bicarbonate (2 x 100mL), dried on magnesium sulfate, filtered and concentrated to dryness. Purification by column chromatography on silica gel (gradient: 20% ethyl acetate in hexanes to 100% ethyl acetate) afforded (3aR,6aS)-5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione as a yellow oil (3.5 g, 56%).

**Step 2. Preparation of Racemic (cis) (1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol 302**

To a cooled (0°C) solution of (3aR,6aS)-5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione (3.5 g, 13.4 mmol) in anhydrous diethyl ether (50 mL) was added slowly lithium aluminum hydride pellets (1.5 g, 40 mmol) over three portions. The solution was stirred overnight warming to room temperature as the ice water bath melted. Upon completion, the reaction was cooled to 0°C and very slowly quenched with 1.5 mL of 5M NaOH followed by 1.5 mL of water. Stir for 30 minutes then add magnesium sulfate and filter. The filtrate was concentrated to afford ((3R,4S)-1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol as a colorless oil (2.7 g).

**Step 3. Preparation of Racemic (cis) (3,4-Dimethylpyrrolidine-3,4-diyl)dimethanol 303**

To a solution of ((3R,4S)-1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol (10 g, 40 mmol) in methanol (10 mL) was added 10% palladium on activated charcoal wet (1 g). The solution was stirred vigorously under a hydrogen atmosphere for 16 hours. Upon completion the solution was filtered through Celite, and concentrated to dryness to afford ((3R,4S)-3,4-Dimethylpyrrolidine-3,4-diyl)dimethanol as a colorless solid (5.5 g, 86%).

**Step 4. Preparation of Racemic (cis) Methyl 10-(3,4-bis(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 304**

A solution of **3** (1.3 g, 8.2 mmol) and monomethyl sebacate (1.8 g, 8.2 mmol) in $CH_2Cl_2$ (100mL) was treated with HBTU (3.41g, 9.02mmol) and Hunig's base (5.71mL, 32.8mmol).

After stirring overnight the mixture was washed with $NaHCO_3$ (sat. aq.), water and brine, then dried ($MgSO_4$), filtered and concentrated. The crude material was subjected to chromatography (gradient: 0% $CH_3OH$-$CH_2Cl_2$ to 20%) to yield 4 (1.8g, 61%).

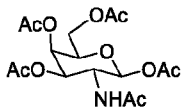
**Step 5. Preparation of Racemic (cis) Methyl 10-(3-((bis(4-methoxyphenyl)(phenyl)-methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 305**

A solution of **304** (1.8 g, 5.0 mmol) and 4,4′-Dimethoxytrityl chloride (1.7 g, 5.0 mmol) in pyridine (180mL) was stirred overnight. The pyridine was then removed under reduced pressure and the crude material was subjected to chromatography (gradient: 0% $CH_3OH$-$CH_2Cl_2$ to 10%) to yield **5** (1.4 g, 42%) as a yellow oil.

**Step 6. Preparation of Racemic (cis) Lithium 10-(3-((bis(4-methoxyphenyl)-(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 306**

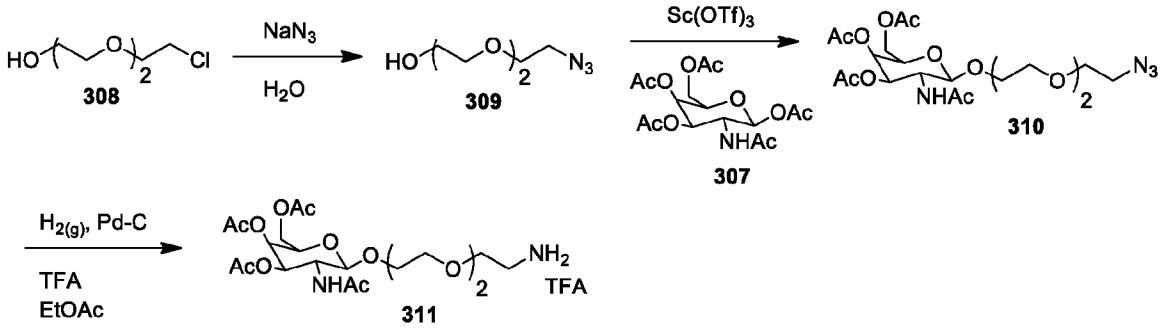
To a solution of compound **305** (3.0 g, 4.6 mmol) in THF (50 mL) and water (50 mL) was added lithium hydroxide (121 mg, 5.0 mmol). The solution was stirred for 4 hours at room temperature then concentrated to remove the THF. The remaining aqueous solution was freeze dried overnight to afford a pale pink solid (2.9 g, quantitative). Compound **306** was prepared as a mixture of two *cis*-diastereomers.

**Scheme 51 Synthesis of peracetylated galactosamine 307**




D-Galactosamine hydrochloride (250 g, 1.16 mol) in pyridine (1.5 L) was treated with acetic anhydride (1.25 L, 13.2 mol) over 45 minutes. After stirring overnight the reaction mixture was divided into three 1 L portions. Each 1 L portion was poured into 3 L of ice water and mixed for one hour. After mixing the solids were filtered off, combined, frozen over liquid nitrogen and then lyophilized for five days to yield peracetylated galactosamine **7** (369.4 g, 82%) as a white solid. Rf (0.58, 10% MeOH-$CH_2Cl_2$).

**Scheme 52 Synthesis of GalNAc monomer**




**Step 1 Preparation of compound 309**

A solution of 2-[2-(2-chloroethoxy)]ethanol **308** (100g, 593mmol) in water (1L) was treated with $NaN_3$ (77g, 1.19mol) and heated (90°C). After stirring (72 hours) the solution was cooled (RT) and extracted (4x) with $CH_2Cl_2$. The combined organics were washed with brine, dried ($MgSO_4$), filtered, concentrated and used without further processing. Compound **9** (88.9g, 86%) was obtained as a pale yellow oil.

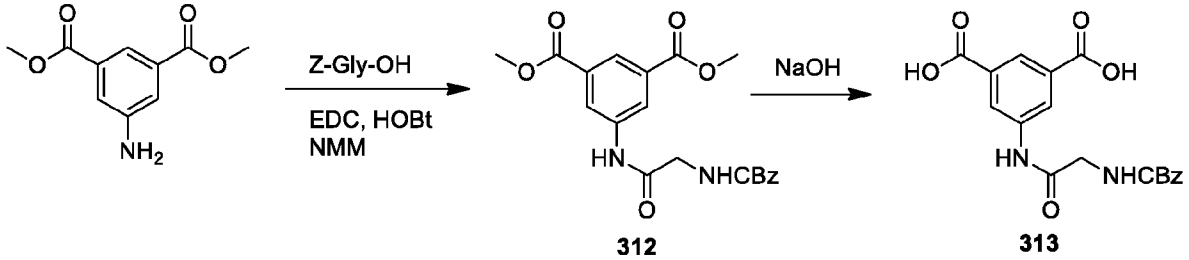
**Step 2 Preparation of compound 310**

A solution of **7** (2.76g, 7.1mmol) and **309** (1.37g, 7.8mmol) in 1,2-dichloroethane (40mL) was treated with $Sc(OTf)_3$ (174mg, 0.36mmol) and heated (85°C). After stirring (2 hours) the mixture was cooled (RT) and quenched by the addition of TEA (4mL) and concentrated. The crude material was subjected to chromatography to yield **310** (3.03g, 85%) as a pale yellow foam.

**Step 3 Preparation of compound 311**

A solution of **310** (3.02g, 5.99mmol) and Pd/C (300mg, 10% Pd loading - wet support) in EtOAc (30mL) was treated with TFA (576µL, 7.5mmol). The reaction mixture was purged with hydrogen gas (45min) then purged with nitrogen gas (10min), then filtered through celite. The filtrate was concentrated and then subjected to chromatography to yield **311** (2.67g, 75%) as a brown foam.

**Scheme 53 Synthesis of aromatic core**



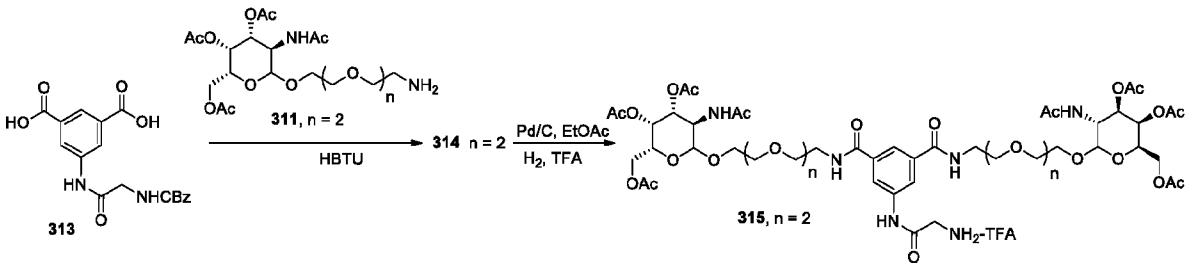

**Step 1. Preparation of Dimethyl 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)-isophthalate 312**

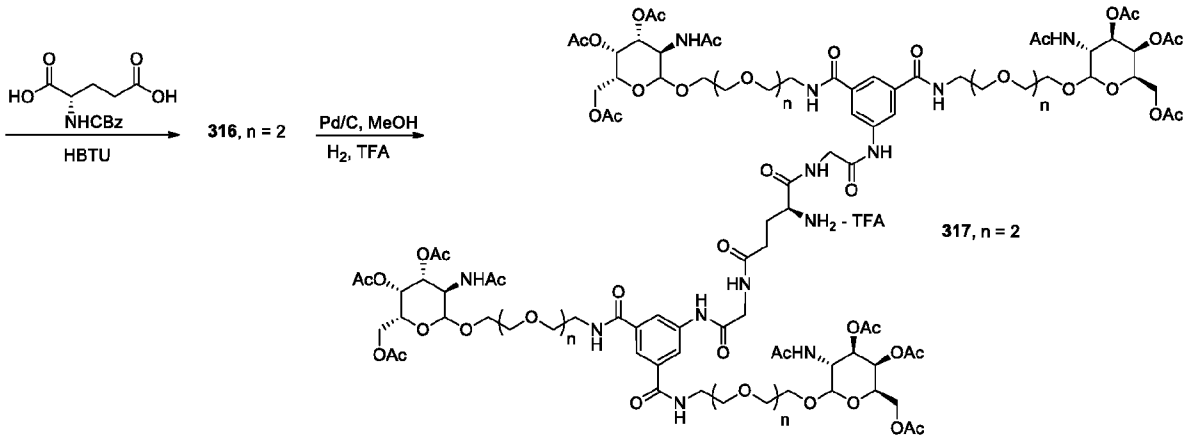
A solution of dimethyl 5-aminoisophthalate (5 g, 24 mmol), Z-Gly-OH (5 g, 24 mmol), EDC (5 g, 26.3 mmol), HOBt (3.6 g, 26.3 mmol), NMM (2.9 mL, 26.3 mmol) in DMF (50 mL) was stirred overnight at room temperature. Upon completion, the reaction mixture was diluted with ethyl acetate (250 mL) and washed with each 1M HCl (2 x 100 mL), saturated sodium bicarbonate (1 x 100 mL) and brine (2 x 100 mL). Dry on magnesium sulfate, filter and concentrate to dryness to afford Dimethyl 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)-acetamido)isophthalate as a colorless solid (7.2 g, 79%).

**Step 2. Preparation of 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)isophthalic acid 313**

To a solution of methyl 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)isophthalate (7.2 g) in methanol (25 mL) and THF (25 mL) was added 1M NaOH (25 mL). The solution was stirred at room temperature for 2 hours then concentrated to remove THF and MeOH. The aqueous solution remaining was diluted with water (75 mL), cooled on an ice water bath and acidified to pH = 1 with 6M HCl. The solid was filtered and washed with water (3 x 100 mL). The solid was freeze dried to afford 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)-isophthalic acid (6.9 g, quantitative) .

**Scheme 54: Preparation of tetramer**






**Step 1 Preparation of compound 314**

A solution of **313** (2.09g, 5.6mmol) and **311** (8.34g, 14.07mmol) in $CH_2Cl_2$ (150mL) was treated with HBTU (6.4g, 16.9mmol) and Hunig's base (7.35mL, 42.2mmol). After stirring (overnight) the reaction mixture was poured into $NaHCO_3$ (sat. aq.) then washed with water and brine, dried ($MgSO_4$), filtered and concentrated. The crude material was subjected to chromatography (gradient 1-12% $CH_3OH$-$CH_2Cl_2$) to yield 6 (3.97g, 55%) as a pale yellow foam.

**Step 2 Preparation of compound 315**

Compound **314** (3.92g, 3.07mmol), Pd/C (400mg, 10% loading – wet support) and trifluoroacetic acid (308µL, 4mmol) was purged with $H_2$. After stirring under $H_2$ (overnight), the mixture was purged with $N_2$ (15-20 min) then filtered through celite and concentrated. The crude material was subjected to chromatography to yield 7 (3.36g, 86%) as a white to cream colored foam.

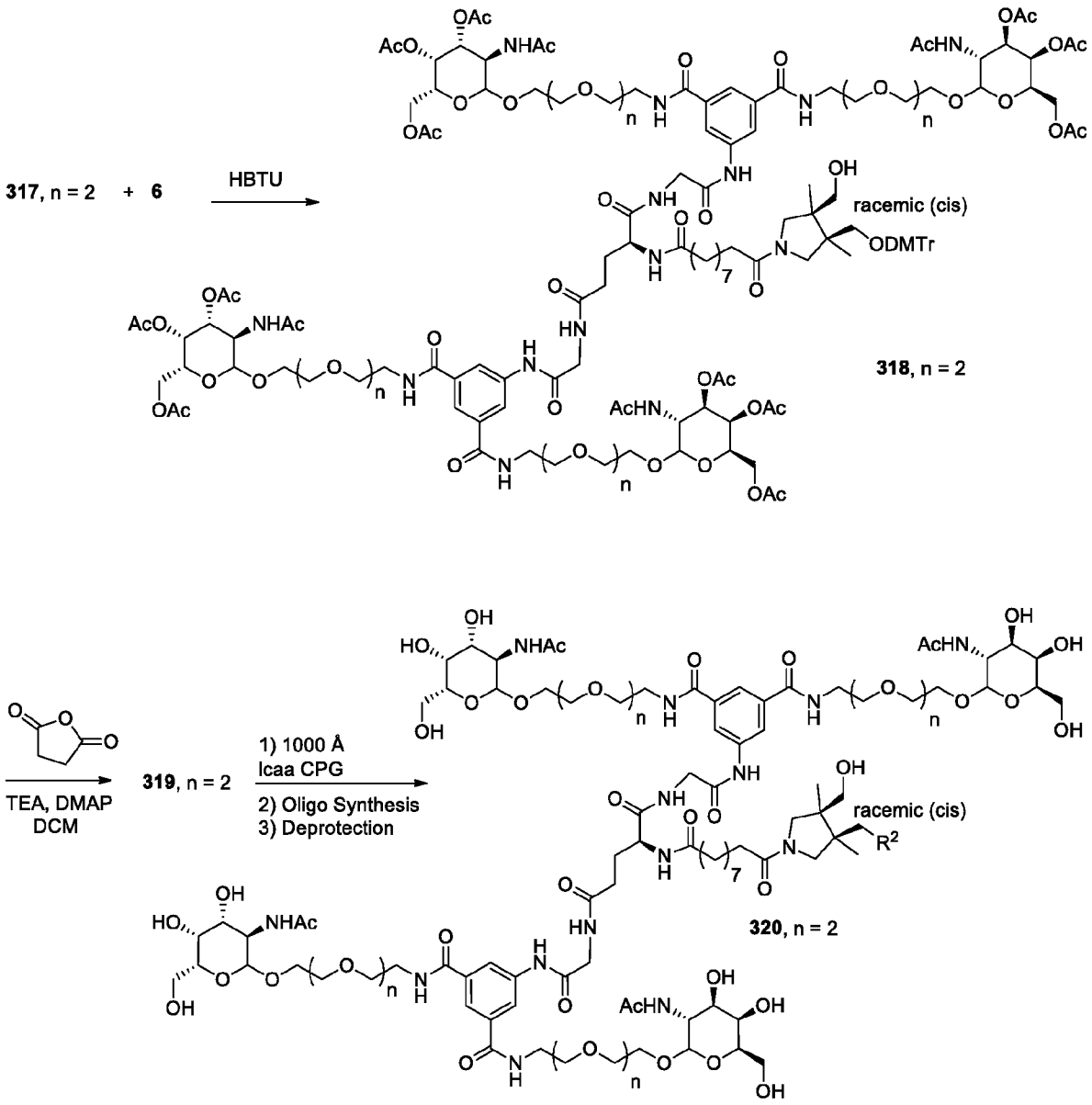
**Step 3 Preparation of compound 316**

Compound **316** was prepared in the same fashion as **314**, from Z-glutamic acid (306mg, 1.09mmol) and **315** (3.3g, 2.6mmol). Yield 1.66g, 60%.

**Step 4 Preparation of compound 317**

Compound **317** was prepared in the same fashion as **315**. Yield 1.65g, Quant.

**Scheme 55 Preparation of complete conjugate**




**Step 1 Preparation of compound 318**

A solution of **317** (1.91g, 0.75mmol) in $CH_2Cl_2$ (100mL) was treated first with Hunig's base (392μL, 2.25mmol) then **6** (a mixture of two *cis*-diastereomers, 509mg, 0.79mmol) followed by HBTU (356mg, 0.94mmol). After stirring (overnight) the solution was poured into $NaHCO_3$ (sat. aq.) then washed with water and brine, dried ($MgSO_4$), filtered and concentrated. The crude material was subjected to chromatography to yield **318** (1.19g, 52%) as a white foam.

**Step 2 Preparation of compound 319**

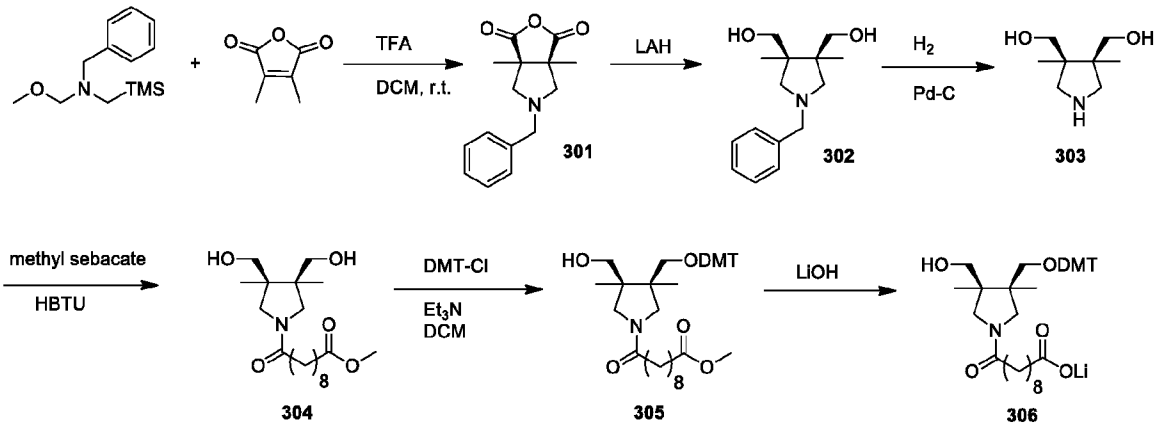
A solution of **318** (1.19g, 0.39mmol) in 1,2 dichloroethane (100mL) was treated with TEA (542µL, 3.9mmol), DMAP (238mg, 1.95mmol) and succinic anhydride (195mg, 1.95mmol) and heated (85°C). After stirring (2.5 hours) the solution was removed from heat and treated with $CH_3OH$ (10mL) and allowed to stir (1 hour). After stirring the mixture was poured into $NaHCO_3$ (sat. aq.) then washed with brine, dried ($MgSO_4$), filtered and concentrated. The residue obtained was used without further processing. Yield = 1.4g, Quant.

**Step 3 Preparation of conjugate 320**

The succinate **319** was loaded onto 1000Å LCAA (long chain aminoalkyl) CPG (control pore glass) using standard amide coupling chemistry. A solution of diisopropylcarbodiimide (52.6 µmol), N-hydroxy succinimide (0.3 mg, 2.6 µmol) and pyridine (10 µL) in anhydrous acetonitrile (0.3 mL) was added to **319** (20.6 mg, 8 µmol) in anhydrous dichloromethane (0.2 mL). This mixture was added to LCAA CPG (183 mg). The suspension was gently mixed overnight at room temperature. Upon disappearance of **319** (HPLC), the reaction mixture was filtered and the CPG was washed with 1 mL of each dichloromethane, acetonitrile, a solution of 5% acetic anhydride / 5% N-methylimidazole / 5% pyridine in THF, then THF, acetonitrile and dichloromethane. The CPG was then dried overnight under high vacuum. Loading was determined by standard DMTr assay by UV/Vis (504 nm) to be 19 µmol/g. The resulting GalNAc loaded CPG solid support was employed in automated oligonucleotide synthesis using standard procedures. Nucleotide deprotection followed by removal from the solid support (with concurrent galactosamine acetate deprotection) afforded the GalNAc-oligonucleotide conjugate **320**.

## Example 26 Synthesis of conjugate 520

**Scheme 56 Preparation of activated linker**




**Step 1. Preparation of Racemic (cis) 5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione 301**

To a cooled solution (0°C) of 3,4-dimethylfuran-2,5-dione (3 g, 24 mmol) and N-benzyl-1-methoxy-N-((trimethylsilyl)methyl)methanamine (7 g, 29.8 mmol) in dichloromethane (75 mL) was slowly added trifluoroacetic acid (75 µL). Stir overnight allowing the solution to slowly warm to room temperature as the ice bath melted. The reaction mixture was concentrated to dryness, dissolved in ethyl acetate (100 mL), washed with saturated sodium bicarbonate (2 x 100mL), dried on magnesium sulfate, filtered and concentrated to dryness. Purification by column chromatography on silica gel (gradient: 20% ethyl acetate in hexanes to 100% ethyl acetate) afforded (3aR,6aS)-5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione as a yellow oil (3.5 g, 56%).

**Step 2. Preparation of Racemic (cis) (1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol 302**

To a cooled (0°C) solution of (3aR,6aS)-5-Benzyl-3a,6a-dimethyltetrahydro-1H-furo[3,4-c]pyrrole-1,3(3aH)-dione (3.5 g, 13.4 mmol) in anhydrous diethyl ether (50 mL) was added slowly lithium aluminum hydride pellets (1.5 g, 40 mmol) over three portions. The solution was stirred overnight warming to room temperature as the ice water bath melted. Upon completion, the reaction was cooled to 0°C and very slowly quenched with 1.5 mL of 5M NaOH followed by 1.5 mL of water. Stir for 30 minutes then add magnesium sulfate and filter. The filtrate was concentrated to afford ((3R,4S)-1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol as a colorless oil (2.7 g).

**Step 3. Preparation of Racemic (cis) (3,4-Dimethylpyrrolidine-3,4-diyl)dimethanol 303**

To a solution of ((3R,4S)-1-Benzyl-3,4-dimethylpyrrolidine-3,4-diyl)dimethanol (10 g, 40 mmol) in methanol (10 mL) was added 10% palladium on activated charcoal wet (1 g). The solution was stirred vigorously under a hydrogen atmosphere for 16 hours. Upon completion the solution was filtered through Celite, and concentrated to dryness to afford ((3R,4S)-3,4-Dimethylpyrrolidine-3,4-diyl)dimethanol as a colorless solid (5.5 g, 86%).

**Step 4. Preparation of Racemic (cis) Methyl 10-(3,4-bis(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 304**

A solution of **3** (1.3 g, 8.2 mmol) and monomethyl sebacate (1.8 g, 8.2 mmol) in $CH_2Cl_2$ (100mL) was treated with HBTU (3.41g, 9.02mmol) and Hunig's base (5.71mL, 32.8mmol). After stirring overnight the mixture was washed with $NaHCO_3$ (sat. aq.), water and brine, then dried ($MgSO_4$), filtered and concentrated. The crude material was subjected to chromatography (gradient: 0% $CH_3OH$-$CH_2Cl_2$ to 20%) to yield 4 (1.8g, 61%).

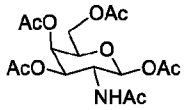
**Step 5. Preparation of Racemic (cis) Methyl 10-(3-((bis(4-methoxyphenyl)(phenyl)-methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 305**

A solution of **304** (1.8 g, 5.0 mmol) and 4,4′-Dimethoxytrityl chloride (1.7 g, 5.0 mmol) in pyridine (180mL) was stirred overnight. The pyridine was then removed under reduced pressure and the crude material was subjected to chromatography (gradient: 0% $CH_3OH$-$CH_2Cl_2$ to 10%) to yield **5** (1.4 g, 42%) as a yellow oil.

**Step 6. Preparation of Racemic (cis) Lithium 10-(3-((bis(4-methoxyphenyl)-(phenyl)methoxy)methyl)-4-(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-10-oxodecanoate 306**

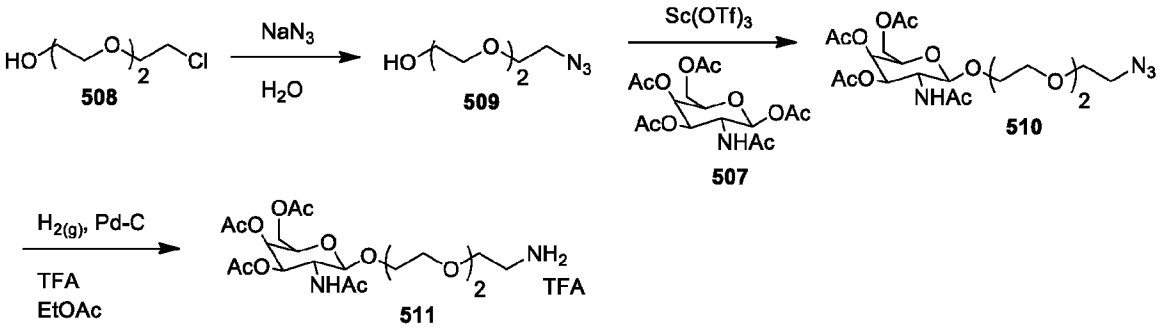
To a solution of compound **305** (3.0 g, 4.6 mmol) in THF (50 mL) and water (50 mL) was added lithium hydroxide (121 mg, 5.0 mmol). The solution was stirred for 4 hours at room temperature then concentrated to remove the THF. The remaining aqueous solution was freeze dried overnight to afford a pale pink solid (2.9 g, quantitative). Compound **306** was prepared as a mixture of two *cis*-diastereomers.

**Scheme 57 Synthesis of peracetylated galactosamine 507**



Galactosamine hydrochloride (250 g, 1.16 mol) in pyridine (1.5 L) is treated with acetic anhydride (1.25 L, 13.2 mol) over 45 minutes. After stirring overnight the reaction mixture is divided into three 1 L portions. Each 1 L portion is poured into 3 L of ice water and mixed for one hour. After mixing the solids are filtered off, combined, frozen over liquid nitrogen and then lyophilized for five days to yield peracetylated galactosamine **507** (369.4 g, 82%) as a white solid. Rf (0.58, 10% MeOH-$CH_2Cl_2$).

**Scheme 58 Synthesis of GalNAc monomer**



**Step 1 Preparation of compound 509**

A solution of 2-[2-(2-chloroethoxy)]ethanol **508** (100g, 593mmol) in water (1L) is treated with $NaN_3$ (77g, 1.19mol) and heated (90°C). After stirring (72 hours) the solution is cooled (RT) and extracted (4x) with $CH_2Cl_2$. The combined organics are washed with brine, dried ($MgSO_4$), filtered, concentrated and used without further processing. Compound **509** (88.9g, 86%) is obtained as a pale yellow oil.

**Step 2 Preparation of compound 510**

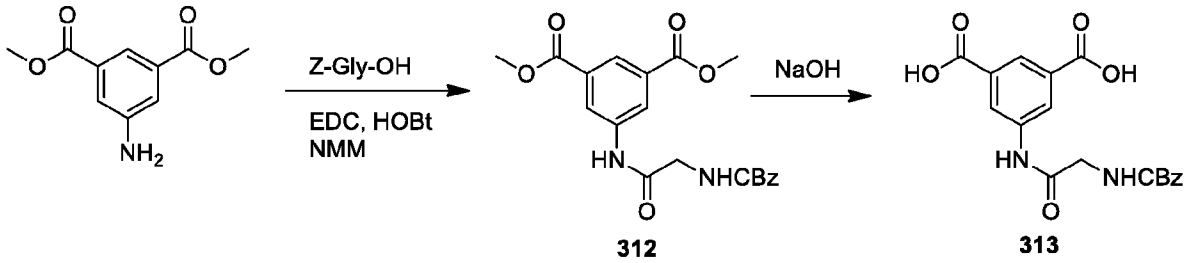
A solution of **507** (2.76g, 7.1mmol) and **509** (1.37g, 7.8mmol) in 1,2-dichloroethane (40mL) is treated with $Sc(OTf)_3$ (174mg, 0.36mmol) and heated (85°C). After stirring (2 hours) the mixture is cooled (RT) and quenched by the addition of TEA (4mL) and concentrated. The crude material is subjected to chromatography to yield **510** (3.03g, 85%) as a pale yellow foam.

**Step 3 Preparation of compound 511**

A solution of **510** (3.02g, 5.99mmol) and Pd/C (300mg, 10% Pd loading - wet support) in EtOAc (30mL) is treated with TFA (576µL, 7.5mmol). The reaction mixture is purged with

hydrogen gas (45min) then purged with nitrogen gas (10min), then filtered through celite. The filtrate is concentrated and then subjected to chromatography to yield **511** (2.67g, 75%) as a brown foam.

**Scheme 59 Synthesis of aromatic core**




312 313

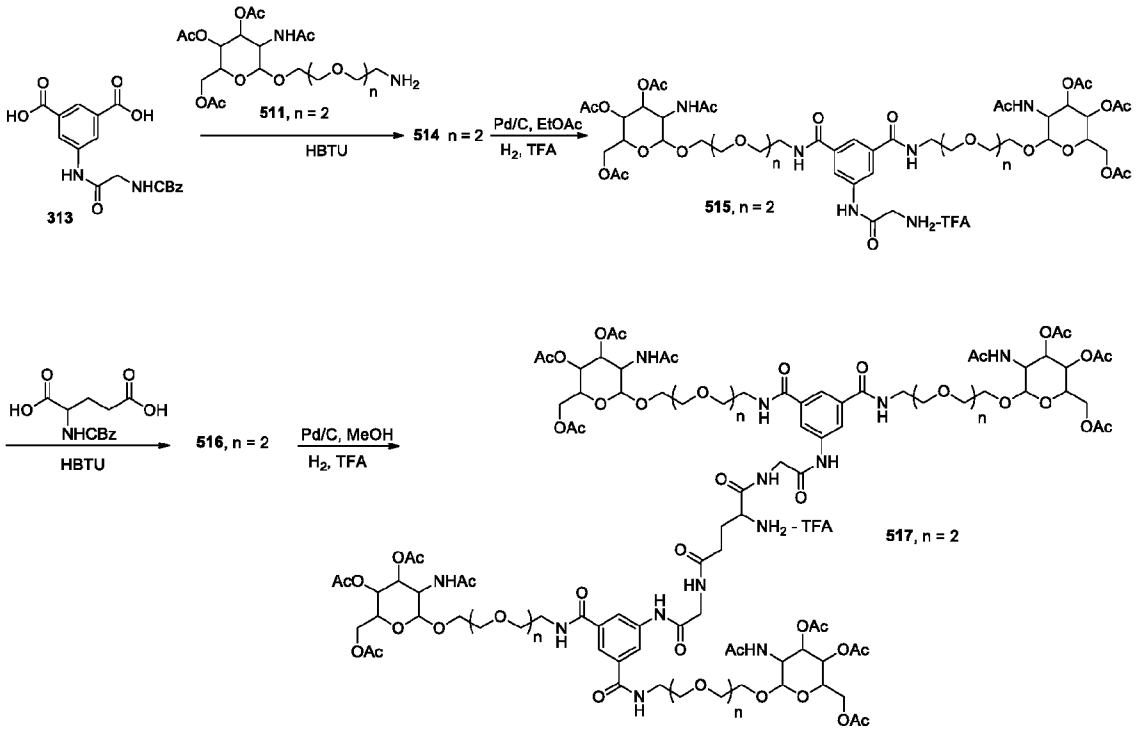
**Step 1. Preparation of Dimethyl 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)-isophthalate 312**

A solution of dimethyl 5-aminoisophthalate (5 g, 24 mmol), Z-Gly-OH (5 g, 24 mmol), EDC (5 g, 26.3 mmol), HOBt (3.6 g, 26.3 mmol), NMM (2.9 mL, 26.3 mmol) in DMF (50 mL) was stirred overnight at room temperature. Upon completion, the reaction mixture was diluted with ethyl acetate (250 mL) and washed with each 1M HCl (2 x 100 mL), saturated sodium bicarbonate (1 x 100 mL) and brine (2 x 100 mL). Dry on magnesium sulfate, filter and concentrate to dryness to afford Dimethyl 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)-acetamido)isophthalate as a colorless solid (7.2 g, 79%).

**Step 2. Preparation of 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)isophthalic acid 313**

To a solution of methyl 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)isophthalate (7.2 g) in methanol (25 mL) and THF (25 mL) was added 1M NaOH (25 mL). The solution was stirred at room temperature for 2 hours then concentrated to remove THF and MeOH. The aqueous solution remaining was diluted with water (75 mL), cooled on an ice water bath and acidified to pH = 1 with 6M HCl. The solid was filtered and washed with water (3 x 100 mL). The solid was freeze dried to afford 5-(2-((2-oxo-2-phenyl-1λ²-ethyl)amino)acetamido)-isophthalic acid (6.9 g, quantitative) .

**Scheme 60: Preparation of tetramer**




**Step 1 Preparation of compound 514**

A solution of **313** (2.09g, 5.6mmol) and **511** (8.34g, 14.07mmol) in $CH_2Cl_2$ (150mL) is treated with HBTU (6.4g, 16.9mmol) and Hunig's base (7.35mL, 42.2mmol). After stirring (overnight) the reaction mixture is poured into $NaHCO_3$ (sat. aq.) then washed with water and brine, dried ($MgSO_4$), filtered and concentrated. The crude material is subjected to chromatography (gradient 1-12% $CH_3OH$-$CH_2Cl_2$) to yield 6 (3.97g, 55%) as a pale yellow foam.

**Step 2 Preparation of compound 515**

Compound **514** (3.92g, 3.07mmol), Pd/C (400mg, 10% loading – wet support) and trifluoroacetic acid (308μL, 4mmol) is purged with $H_2$. After stirring under $H_2$ (overnight), the mixture is purged with $N_2$ (15-20 min) then filtered through celite and concentrated. The crude material is subjected to chromatography to yield 7 (3.36g, 86%) as a white to cream colored foam.

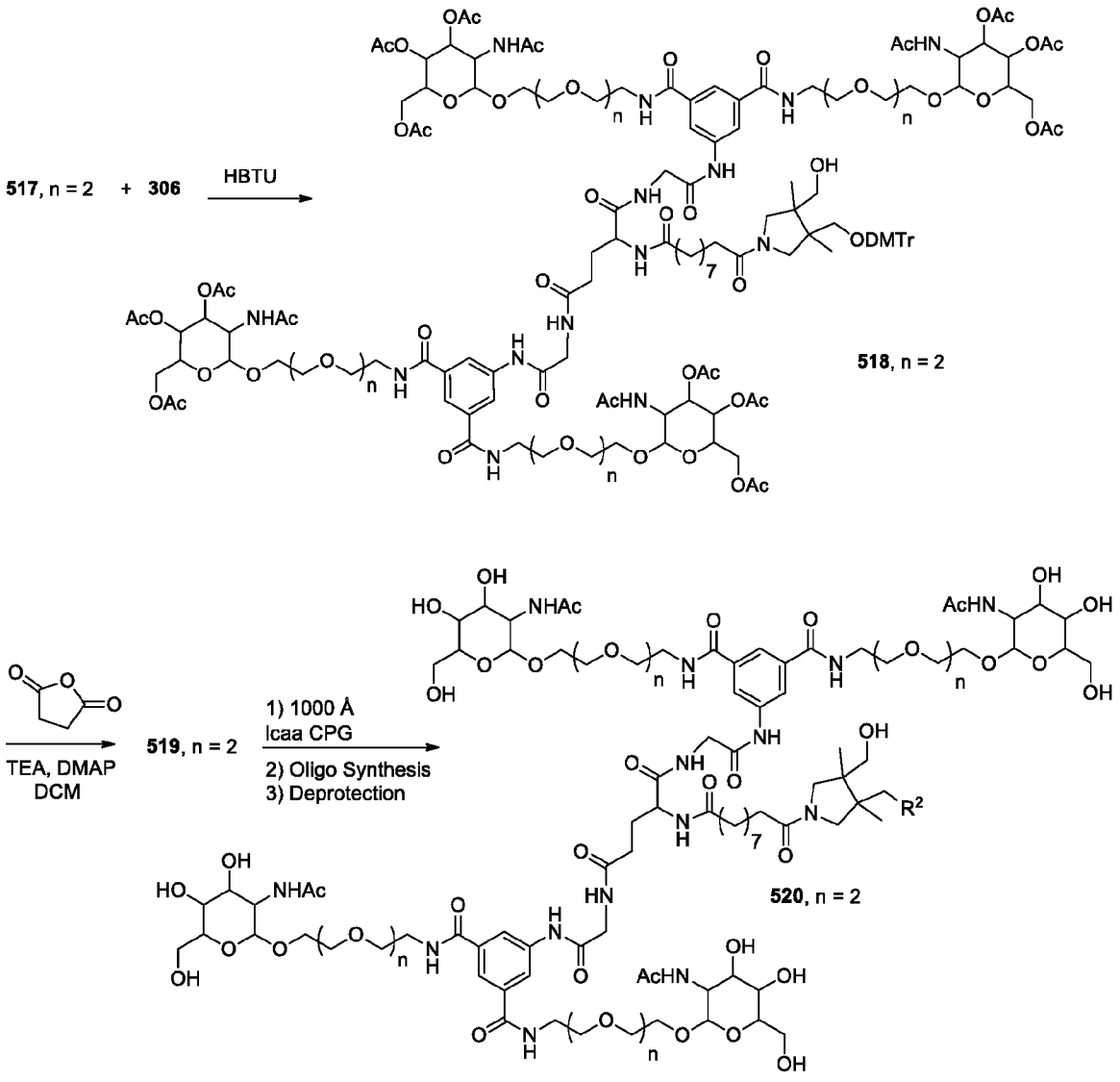
**Step 3 Preparation of compound 516**

Compound **516** is prepared in the same fashion as **514**, from Z-glutamic acid (306mg, 1.09mmol) and **515** (3.3g, 2.6mmol). Yield 1.66g, 60%.

**Step 4 Preparation of compound 517**

Compound **517** is prepared in the same fashion as **515**. Yield 1.65g, Quant.

**Scheme 61 Preparation of complete conjugate**




**Step 1 Preparation of compound 518**

A solution of **517** (1.91g, 0.75mmol) in $CH_2Cl_2$ (100mL) is treated first with Hunig's base (392μL, 2.25mmol) then **306** (a mixture of two *cis*-diastereomers, 509mg, 0.79mmol) followed by HBTU (356mg, 0.94mmol). After stirring (overnight) the solution was poured into $NaHCO_3$ (sat. aq.) then washed with water and brine, dried ($MgSO_4$), filtered and concentrated. The crude material is subjected to chromatography to yield **518** (1.19g, 52%) as a white foam.

**Step 2 Preparation of compound 519**

A solution of **518** (1.19g, 0.39mmol) in 1,2 dichloroethane (100mL) is treated with TEA (542µL, 3.9mmol), DMAP (238mg, 1.95mmol) and succinic anhydride (195mg, 1.95mmol) and heated (85°C). After stirring (2.5 hours) the solution is removed from heat and treated with $CH_3OH$ (10mL) and allowed to stir (1 hour). After stirring the mixture is poured into $NaHCO_3$ (sat. aq.) then washed with brine, dried ($MgSO_4$), filtered and concentrated. The residue obtained is used without further processing. Yield = 1.4g, Quant.

**Step 3 Preparation of conjugate 520**

The succinate **519** is loaded onto 1000Å LCAA (long chain aminoalkyl) CPG (control pore glass) using standard amide coupling chemistry. A solution of diisopropylcarbodiimide (52.6 µmol), N-hydroxy succinimide (0.3 mg, 2.6 µmol) and pyridine (10 µL) in anhydrous acetonitrile (0.3 mL) is added to **519** (20.6 mg, 8 µmol) in anhydrous dichloromethane (0.2 mL). This mixture is added to LCAA CPG (183 mg). The suspension was gently mixed overnight at room temperature. Upon disappearance of **519** (HPLC), the reaction mixture is filtered and the CPG is washed with 1 mL of each dichloromethane, acetonitrile, a solution of 5% acetic anhydride / 5% N-methylimidazole / 5% pyridine in THF, then THF, acetonitrile and dichloromethane. The CPG is then dried overnight under high vacuum. Loading was determined by standard DMTr assay by UV/Vis (504 nm) to be 19 µmol/g. The resulting GalNAc loaded CPG solid support is employed in automated oligonucleotide synthesis using standard procedures. Nucleotide deprotection followed by removal from the solid support (with concurrent galactosamine acetate deprotection) affords the GalNAc-oligonucleotide conjugate **520**.

**Example 27. Two Way Combinations of siRNA Molecules**

Certain embodiments of the present invention provide the use of combinations of two of the siRNA molecules described herein, *e.g.*, as a combination in a composition or a nucleic acid-lipid particle, *e.g.*, a combination of two of siRNA molecules 1m-80m or a combination of two of siRNA molecules 101-137.

**Example 28. Three Way Combinations of siRNA Molecules**

Certain embodiments of the present invention provide the use of combinations of three of the siRNA molecules described herein, *e.g.*, as a combination in a composition or a nucleic acid-lipid particle, *e.g.*, a combination of three of siRNA molecules 1m-80m or a combination of

three of siRNA molecules 101-137. While not intending to be limited to these combinations, certain combinations of three siRNA molecules include the following.

By way of example, the three way siRNA combinations of siRNAs 1m thru 15m are: 1m-2m-3m;1m-2m-4m;1m-2m-5m;1m-2m-6m;1m-2m-7m;1m-2m-8m;1m-2m-9m;1m-2m-10m;1m-2m-11m;1m-2m-12m;1m-2m-13m;1m-2m-14m;1m-2m-15m;1m-3m-4m;1m-3m-5m;1m-3m-6m;1m-3m-7m;1m-3m-8m;1m-3m-9m;1m-3m-10m;1m-3m-11m;1m-3m-12m;1m-3m-13m;1m-3m-14m;1m-3m-15m;1m-4m-5m;1m-4m-6m;1m-4m-7m;1m-4m-8m;1m-4m-9m;1m-4m-10m;1m-4m-11m;1m-4m-12m;1m-4m-13m;1m-4m-14m;1m-4m-15m;1m-5m-6m;1m-5m-7m;1m-5m-8m;1m-5m-9m;1m-5m-10m;1m-5m-11m;1m-5m-12m;1m-5m-13m;1m-5m-14m;1m-5m-15m;1m-6m-7m;1m-6m-8m;1m-6m-9m;1m-6m-10m;1m-6m-11m;1m-6m-12m;1m-6m-13m;1m-6m-14m;1m-6m-15m;1m-7m-8m;1m-7m-9m;1m-7m-10m;1m-7m-11m;1m-7m-12m;1m-7m-13m;1m-7m-14m;1m-7m-15m;1m-8m-9m;1m-8m-10m;1m-8m-11m;1m-8m-12m;1m-8m-13m;1m-8m-14m;1m-8m-15m;1m-9m-10m;1m-9m-11m;1m-9m-12m;1m-9m-13m;1m-9m-14m;1m-9m-15m;1m-10m-11m;1m-10m-12m;1m-10m-13m;1m-10m-14m;1m-10m-15m;1m-11m-12m;1m-11m-13m;1m-11m-14m;1m-11m-15m;1m-12m-13m;1m-12m-14m;1m-12m-15m;1m-13m-14m;1m-13m-15m;1m-14m-15m;2m-3m-4m;2m-3m-5m;2m-3m-6m;2m-3m-7m;2m-3m-8m;2m-3m-9m;2m-3m-10m;2m-3m-11m;2m-3m-12m;2m-3m-13m;2m-3m-14m;2m-3m-15m;2m-4m-5m;2m-4m-6m;2m-4m-7m;2m-4m-8m;2m-4m-9m;2m-4m-10m;2m-4m-11m;2m-4m-12m;2m-4m-13m;2m-4m-14m;2m-4m-15m;2m-5m-6m;2m-5m-7m;2m-5m-8m;2m-5m-9m;2m-5m-10m;2m-5m-11m;2m-5m-12m;2m-5m-13m;2m-5m-14m;2m-5m-15m;2m-6m-7m;2m-6m-8m;2m-6m-9m;2m-6m-10m;2m-6m-11m;2m-6m-12m;2m-6m-13m;2m-6m-14m;2m-6m-15m;2m-7m-8m;2m-7m-9m;2m-7m-10m;2m-7m-11m;2m-7m-12m;2m-7m-13m;2m-7m-14m;2m-7m-15m;2m-8m-9m;2m-8m-10m;2m-8m-11m;2m-8m-12m;2m-8m-13m;2m-8m-14m;2m-8m-15m;2m-9m-10m;2m-9m-11m;2m-9m-12m;2m-9m-13m;2m-9m-14m;2m-9m-15m;2m-10m-11m;2m-10m-12m;2m-10m-13m;2m-10m-14m;2m-10m-15m;2m-11m-12m;2m-11m-13m;2m-11m-14m;2m-11m-15m;2m-12m-13m;2m-12m-14m;2m-12m-15m;2m-13m-14m;2m-13m-15m;2m-14m-15m;3m-4m-5m;3m-4m-6m;3m-4m-7m;3m-4m-8m;3m-4m-9m;3m-4m-10m;3m-4m-11m;3m-4m-12m;3m-4m-13m;3m-4m-14m;3m-4m-15m;3m-5m-6m;3m-5m-7m;3m-5m-8m;3m-5m-9m;3m-5m-10m;3m-5m-11m;3m-5m-12m;3m-5m-13m;3m-5m-14m;3m-5m-15m;3m-6m-7m;3m-6m-8m;3m-6m-9m;3m-6m-10m;3m-6m-11m;3m-6m-12m;3m-6m-13m;3m-6m-14m;3m-6m-15m;3m-7m-8m;3m-7m-9m;3m-7m-10m;3m-7m-11m;3m-7m-12m;3m-7m-13m;3m-7m-14m;3m-7m-15m;3m-8m-9m;3m-8m-10m;3m-8m-11m;3m-8m-12m;3m-8m-13m;3m-8m-14m;3m-8m-15m;3m-9m-10m;3m-9m-11m;3m-9m-12m;3m-9m-13m;3m-9m-14m;3m-9m-15m;3m-10m-

11m;3m-10m-12m;3m-10m-13m;3m-10m-14m;3m-10m-15m;3m-11m-12m;3m-11m-13m;3m-11m-14m;3m-11m-15m;3m-12m-13m;3m-12m-14m;3m-12m-15m;3m-13m-14m;3m-13m-15m;3m-14m-15m;4m-5m-6m;4m-5m-7m;4m-5m-8m;4m-5m-9m;4m-5m-10m;4m-5m-11m;4m-5m-12m;4m-5m-13m;4m-5m-14m;4m-5m-15m;4m-6m-7m;4m-6m-8m;4m-6m-9m;4m-6m-10m;4m-6m-11m;4m-6m-12m;4m-6m-13m;4m-6m-14m;4m-6m-15m;4m-7m-8m;4m-7m-9m;4m-7m-10m;4m-7m-11m;4m-7m-12m;4m-7m-13m;4m-7m-14m;4m-7m-15m;4m-8m-9m;4m-8m-10m;4m-8m-11m;4m-8m-12m;4m-8m-13m;4m-8m-14m;4m-8m-15m;4m-9m-10m;4m-9m-11m;4m-9m-12m;4m-9m-13m;4m-9m-14m;4m-9m-15m;4m-10m-11m;4m-10m-12m;4m-10m-13m;4m-10m-14m;4m-10m-15m;4m-11m-12m;4m-11m-13m;4m-11m-14m;4m-11m-15m;4m-12m-13m;4m-12m-14m;4m-12m-15m;4m-13m-14m;4m-13m-15m;4m-14m-15m;5m-6m-7m;5m-6m-8m;5m-6m-9m;5m-6m-10m;5m-6m-11m;5m-6m-12m;5m-6m-13m;5m-6m-14m;5m-6m-15m;5m-7m-8m;5m-7m-9m;5m-7m-10m;5m-7m-11m;5m-7m-12m;5m-7m-13m;5m-7m-14m;5m-7m-15m;5m-8m-9m;5m-8m-10m;5m-8m-11m;5m-8m-12m;5m-8m-13m;5m-8m-14m;5m-8m-15m;5m-9m-10m;5m-9m-11m;5m-9m-12m;5m-9m-13m;5m-9m-14m;5m-9m-15m;5m-10m-11m;5m-10m-12m;5m-10m-13m;5m-10m-14m;5m-10m-15m;5m-11m-12m;5m-11m-13m;5m-11m-14m;5m-11m-15m;5m-12m-13m;5m-12m-14m;5m-12m-15m;5m-13m-14m;5m-13m-15m;5m-14m-15m;6m-7m-8m;6m-7m-9m;6m-7m-10m;6m-7m-11m;6m-7m-12m;6m-7m-13m;6m-7m-14m;6m-7m-15m;6m-8m-9m;6m-8m-10m;6m-8m-11m;6m-8m-12m;6m-8m-13m;6m-8m-14m;6m-8m-15m;6m-9m-10m;6m-9m-11m;6m-9m-12m;6m-9m-13m;6m-9m-14m;6m-9m-15m;6m-10m-11m;6m-10m-12m;6m-10m-13m;6m-10m-14m;6m-10m-15m;6m-11m-12m;6m-11m-13m;6m-11m-14m;6m-11m-15m;6m-12m-13m;6m-12m-14m;6m-12m-15m;6m-13m-14m;6m-13m-15m;6m-14m-15m;7m-8m-9m;7m-8m-10m;7m-8m-11m;7m-8m-12m;7m-8m-13m;7m-8m-14m;7m-8m-15m;7m-9m-10m;7m-9m-11m;7m-9m-12m;7m-9m-13m;7m-9m-14m;7m-9m-15m;7m-10m-11m;7m-10m-12m;7m-10m-13m;7m-10m-14m;7m-10m-15m;7m-11m-12m;7m-11m-13m;7m-11m-14m;7m-11m-15m;7m-12m-13m;7m-12m-14m;7m-12m-15m;7m-13m-14m;7m-13m-15m;7m-14m-15m;8m-9m-10m;8m-9m-11m;8m-9m-12m;8m-9m-13m;8m-9m-14m;8m-9m-15m;8m-10m-11m;8m-10m-12m;8m-10m-13m;8m-10m-14m;8m-10m-15m;8m-11m-12m;8m-11m-13m;8m-11m-14m;8m-11m-15m;8m-12m-13m;8m-12m-14m;8m-12m-15m;8m-13m-14m;8m-13m-15m;8m-14m-15m;9m-10m-11m;9m-10m-12m;9m-10m-13m;9m-10m-14m;9m-10m-15m;9m-11m-12m;9m-11m-13m;9m-11m-14m;9m-11m-15m;9m-12m-13m;9m-12m-14m;9m-12m-15m;9m-13m-14m;9m-13m-15m;9m-14m-15m;10m-11m-12m;10m-11m-13m;10m-11m-14m;10m-11m-15m;10m-12m-13m;10m-12m-14m;10m-12m-15m;10m-13m-14m;10m-13m-15m;10m-14m-15m;11m-12m-13m;11m-12m-14m;11m-12m-15m;11m-13m-

14m;11m-13m-15m;11m-14m-15m;12m-13m-14m;12m-13m-15m;12m-14m-15m; and 13m-14m-15m.

Other combinations of three different siRNA include, for example, 1m-10m-20m; 1m-10m-30m; 1m-10m-40m; 1m-10m-50m; 1m-10m-60m; 10m-20m-30m; 10m-20m-40m; 10m-20m-50m; 10m-20m-60m; 20m-30m-40m; 20m-30m-50m; 20m-30m-60m; 30m-40m-50m; 30m-40m-60m; 40m-50m-60m; 1m-11m-21m; 1m-11m-31m; 1m-11m-41m; 1m-11m-51m; 1m-11m-61m; 11m-21m-31m; 11m-21m-41m; 11m-21m-51m; 11m-21m-61m; 21m-31m-41m; 21m-31m-51m; 21m-31m-61m; 31m-41m-51m; 31m-41m-61m; 41m-51m-61m; 2m-12m-22m; 2m-12m-32m; 2m-12m-42m; 2m-12m-52m; 2m-12m-62m; 12m-22m-32m; 12m-22m-42m; 12m-22m-52m; 12m-22m-62m; 22m-32m-42m; 22m-32m-52m; 22m-32m-62m; 32m-42m-52m; 32m-42m-62m; 42m-52m-62m.

Other combinations of three different siRNA include, for example, 67m-68m-69m, 67m-68m-73m, 67m-69m-71m, 67m-70m-73m, 67m-71m-73m, 67m-71m-74m, 67m-72m-73m, 68m-69m-70m, 68m-69m-73m, 68m-70m-72m, 68m-71m-73m; 68m-72m-73m, 69m-70m-71m, 69m-70m-73m, 69m-71m-73m, 69m-72m-73m, 70m-71m-72m, 70m-71m-73m, 70m-72m-73m, 71m-72m-73m.

## Example 29. IL-28B polymorphisms in Chronic Hepatitis B subjects

### Study Goal

Several host and viral factors have been found to be associated with differences in HBV clearance or persistence. However, an unexplained variability in treatment outcome still exists, suggesting that the genetic background of the host plays an important role. The literature surrounding the impact of certain SNPs on chronic HBV infection is currently somewhat controversial and not consistent. For example, some groups have found significant correlations between certain SNPs and different responses to PEGylated-interferon (pegIFN) by chronic HBV HBeAg-negative patients. Other groups have looked for, but failed to identify statistically significant correlations.

siRNA-NP2, described below, is an HBV antigen inhibitor having a novel mechanism of action against HBV, which is distinct from that of IFN. With this novel mechanism of action, the correlation between certain SNPs and treatment response to siRNA-NP2 may be stronger or clearer than in the previous studies examining IFN. Thus, the goal of this study was to determinate whether *IL-28B* polymorphisms play a role in subjects with Chronic Hepatitis B (CHB) receiving an HBV antigen inhibitor (e.g., an INF free therapy), such as the siRNA-NP2 formulation described below.

While not intending to be limited by theory, it is hypothesized that individuals with these SNPs have better control of CHB, meaning that even if spontaneous clearance has not yet been achieved, such individuals are closer to reaching the 'tipping point' needed to assert dominant control over the virus. Hepatitis B virus utilizes multiple mechanisms to enforce chronicity of infection, including maintaining high levels of antigen production that are thought to play roles in suppression of host immune responses, amongst other things. In patients with SNPs in, e.g., IL28B, the virus may be less able to upregulate gene expression to compensate for the gene silencing effect of siRNA-NP2, and so therefore, the gene silencing effect of siRNA-NP2 is more clearly detectable in this subset of patients.

**Lipid Nanoparticle Formulation**

The following formulation is an RNA interference product that includes three synthetic double-stranded, small interfering RNAs directed against hepatitis B virus (HBV) messenger RNAs, targeting three distinct sites in the HBV genome. This combination of siRNAs is designed to inhibit viral replication, reduce all HBV transcripts, and lower all viral antigens.

The lipid nanoparticle formulation comprises a mixture of three siRNAs targeting the HBV genome (*see,* siRNA listed below). Specifically, the following lipid nanoparticle (LNP) formulation was used to deliver the HBV siRNAs in the experiments reported herein. This formulation is referred to as **siRNA-NP2** throughout this Example.

| **Component** | **Concentration (mg/ml)** |
|---|---|
| Three siRNA molecules shown below (nominal 1:1:1 ratio) | 2.0 |
| DLin-MP-DMA 3-dilinoleylmethoxy-N,N-dimethylpropan-1-amine **(508)** | 12.8 |
| PEG2000-C-DMA Poly(oxy-1,2-ethanediyl),α-({[2,3-bis(tetradecyloxy) propyl] amino}carbonyl)-ω-methoxy- | 1.7 |
| DSPC 1,2-Distearoyl-*sn*-glycero-3-phosphocholine | 3.3 |
| Cholesterol, synthetic Cholest-5-en-3β-ol, USP-NF | 4.8 |
| Phosphate Buffered Saline | qs |

Abbreviations: qs = *quantum satus*; USP-NF = United States Pharmacopoeia-National Formulary.

A mixture of three siRNAs targeting the HBV genome were used in this experiment. The sequences of the three siRNAs are shown below.

| Name | Sense Sequence (5'-3') | SEQ ID NO. | Antisense Sequence (5'-3') | SEQ ID NO. |
|---|---|---|---|---|
| **67m** | xCrCrGrUmGmUrGrCrArCrUmUrCrGrCmUmUrCrAxUxU | 142 | rUrGrArArGrCrGrArArGrUmGrCrArCrArCmGrGxUxU | 143 |
| **71m** | xCmUmGmGrCrUrCrArGrUrUrUrArCmUrAmGrUrGrUxU | 144 | rCrArCrUrAmGrUrArArArCrUmGrAmGrCrCrArGrUrU | 145 |
| **74m** | xGrCrCmGrAmUrCrCrArUrArCmUmGrCrGmGrArArUxU | 10 | rUrUrCrCrGrCrAmGrUrArUrGmGrArUrCrGmGrCrUrU | 24 |

rN = RNA of base N;
mN = 2'O-methyl modification of base N;
xN = unlocked nucleoside analog moiety of base N

**Methods**

The study was a single-blind, placebo-controlled, multi-dose study in non-cirrhotic, virally suppressed subjects to evaluate safety and efficacy of **siRNA-NP2** initially over 12 weeks in cohorts 1, 2 and 3. Cohort 4 was used to evaluated the safety, PK and anti-viral response of a more frequent dosing regimen (five bi-weekly doses of **siRNA-NP2** with extended monthly dosing out to one year for patients who meet predefined response criteria).

A total of 24 subjects on stable nucleoside therapy were enrolled in the initial 3 cohorts, randomized 3:1 (active vs placebo): Cohort 1, HBeAg(-) at 0.2 mg/kg; Cohort 2, HBeAg(-) at 0.4 mg/kg; Cohort 3, HBeAg(+) at 0.4 mg/kg. Subjects received **siRNA-NP2** as 3 monthly IV doses.

Cohort 4 was an open label multi-dose study in non-cirrhotic HBeAg (-) virally suppressed subjects. A total of 12 subjects on stable nucleoside therapy were enrolled to receive **siRNA-NP2** 0.4 mg/kg bi-weekly for a total of five doses over 8 weeks. Subjects with HBsAg $\leq$1000 IU/mL AND $\geq$1.0 $\log_{10}$ decrease from baseline at Day 71 continued with up to 10 additional monthly doses for a total treatment duration of 48 weeks. Subjects were monitored for safety, PK and HBV markers, and HBV genotype was assessed by line probe assay (INNO-LiPA; Innogenetics N.V., Ghent, Belgium).

The IL-28B associated single nucleotide polymorphism (SNP), rs12979860 (chr. 19q13), was analyzed using a real-time PCR assay. The assay utilized the 5-prime nuclease activity of a thermostable polymerase and unique primers and fluorescent probes to detect the SNP (rs12979860 C/T) in whole blood samples for subjects enrolled on cohort 4.

**Results and Conclusion**

Of 12 subjects, 9 (75%) were male, having a mean age of 48.3 years. Eight of the subjects had the 8 IL28B genotype CC (66%), 8 had HBV genotype C (66%) and the mean baseline HBsAg was 3.43 ($log_{10}$ IU/mL) (Table1). Eleven subjects completed 5 doses of **siRNA-NP2** 0.4 mg/kg. All subjects experienced a reduction in HBsAg from baseline. Seven of 11 (64%) subjects were considered responders prior to or at Day 71; 5/7 (71%) subjects met the criteria by Day 22 (2 doses). Mean HBsAg decline for responders was -1.6 log10 IU/mL at treatment Day 64 with a maximum individual HBsAg decline -2.7 log10 IU/mL; in addition, 5/7 (71%) responders reached HBsAg< 50 IU/mL. Based on this data, it is suggested that the CC genotype for SNP rs12979860 is indicative of a patients demonstrating a higher likelihood of showing a significant response to siRNA-NP2.

**Table 1. HBsAG assessment –Cohort 4**

| Subject ID | HBV Genotype | IL28B | Max HbsAg log decline at any time point | Lower HBsAg During therapy at any time point (IU/ml) |
|---|---|---|---|---|
| 3001-004 | C | CC | -2.66 | 21 |
| 6001-012 | C | CC | -0.65 | 547 |
| 7002-002 | C | CC | -1.73 | 26 |
| 7001-005 | C | CC | -1.01 | 128 |
| 5001-005 | C | CT | -0.86 | 1270 |
| 5001-006 | Could not be amplified | TT | -0.50 | 1360 |
| 7001-006 | Could not be amplified | CC | -1.41 | 49 |
| 7001-003 | B | CC | -1.81 | 16 |
| 5001-008 | C | TT | -0.79 | 2819 |
| 7002-006 | Could not be amplified | CC | -1.23 | 124 |
| 6001-005 | C | | -0.78 | 2974 |
| 6001-020 | C | CC | -2.15 | 21 |

**Table 2. Summary of Demographic and Baseline Characteristics – Cohort 4 Safety**

| Category<br>Stat./Response | Stronger Responders (N=7) | Weaker Responders (N=5) | Total (N=12) |
|---|---|---|---|
| **Age (years)** | | | |
| N | 7 | 5 | 12 |
| Mean (SD) | 52.4 (7.8) | 42.6 (10.3) | 48.3 (9.9) |
| Median | 50 | 45 | 49 |
| Q1, Q3 | 47, 60 | 35, 51 | 44, 55 |
| Min, Max | 42, 64 | 29, 53 | 29, 64 |
| **Age (years), n (%)** | | | |
| <50 years | 3 (42.9) | 3 (60.0) | 6 (50.0) |
| >=50 years | 4 (57.1) | 2 (40.0) | 6 (50.0) |
| **Sex, n (%)** | | | |
| Male | 6 (85.7) | 3 (60.0) | 9 (75.0) |
| Female | 1 (14.3) | 2 (40.0) | 3 (25.0) |
| **Race, n (%)** | | | |
| American Indian or Alaska Native | 0 | 0 | 0 |
| Asian | 5 (71.4) | 1 (20.0) | 6 (50.0) |
| Black or African American | 0 | 2 (40.0) | 2 (16.7) |
| Native Hawaiian or Other Pacific Islander | 0 | 0 | 0 |
| White | 2 (28.6) | 2 (40.0) | 4 (33.3) |
| Other | 0 | 0 | 0 |
| **Ethnicity, n (%)** | | | |
| Hispanic or Latino | 0 | 0 | 0 |
| Not Hispanic or Latino | 7 (100.0) | 5 (100.0) | 12 (100.0) |
| Not Reported | 0 | 0 | 0 |
| Unknown | 0 | 0 | 0 |
| **Weight (kg)** | | | |
| N | 7 | 5 | 12 |
| Mean (SD) | 71.30 (19.74) | 79.44 (14.69) | 74.69 (17.57) |
| Median | 66.0 | 86.0 | 72.9 |
| Q1, Q3 | 57.0, 91.0 | 79.0, 89.0 | 58.7, 89.0 |
| Min, Max | 52.0, 106.0 | 54.2, 89.0 | 52.0, 106.0 |
| **Height (cm)** | | | |
| N | 7 | 5 | 12 |
| Mean (SD) | 172.34 (8.47) | 175.80 (9.15) | 173.78 (8.53) |
| Median | 168.2 | 173.0 | 170.1 |
| Q1, Q3 | 167.0, 183.0 | 168.0, 185.0 | 167.5, 184.0 |
| Min, Max | 163.0, 185.2 | 167.0, 186.0 | 163.0, 186.0 |
| **BMI (kg/m^2)** | | | |
| N | 7 | 5 | 12 |
| Mean (SD) | 23.76 (4.30) | 25.73 (4.87) | 24.58 (4.44) |
| Median | 23.5 | 25.7 | 24.2 |
| Q1, Q3 | 19.6, 26.5 | 24.7, 28.3 | 20.8, 27.4 |
| Min, Max | 19.3, 31.7 | 18.4, 31.5 | 18.4, 31.7 |
| **Baseline ALT (U/L)** | | | |
| N | 7 | 5 | 12 |
| Mean (SD) | 26.71 (19.52) | 36.20 (10.85) | 30.67 (16.57) |
| Median | 16.0 | 31.0 | 29.5 |

| Category<br>Stat./Response | Stronger Responders (N=7) | Weaker Responders (N=5) | Total (N=12) |
|---|---|---|---|
| Q1, Q3 | 14.0, 45.0 | 30.0, 36.0 | 15.5, 40.5 |
| Min, Max | 13.0, 63.0 | 29.0, 55.0 | 13.0, 63.0 |
| **HBV Genotype** | | | |
| B | 1 (14.3) | 0 | 1 (8.3) |
| C | 4 (57.1) | 4 (80.0) | 8 (66.7) |
| Could not be amplified | 2 (28.6) | 1 (20.0) | 3 (25.0) |
| **IL28B Genotype** | | | |
| CC | 7 (100.0) | 1 (20.0) | 8 (66.7) |
| CT | 0 | 1 (20.0) | 1 (8.3) |
| TT | 0 | 2 (40.0) | 2 (16.7) |
| Missing | 0 | 1 ( 20.0) | 1 (8.3) |
| **Baseline HBsAG (log10 IU/mL)** | | | |
| N | 7 | 5 | 12 |
| Mean (SD) | 3.16 (0.31) | 3.80 (0.40) | 3.43 (0.47) |
| Median | 3.0 | 3.8 | 3.2 |
| Q1, Q3 | 2.9, 3.2 | 3.6, 4.1 | 3.0, 3.8 |
| Min, Max | 2.9, 3.8 | 3.2, 4.2 | 2.9, 4.2 |

*Stronger responders: subjects with HBsAg results ≤1000 IU/ml and ≥1 log10 decline in HBsAG from baseline during the biweekly dosing. Weaker responders: subjects with HBsAg results >1000 IU/ml and <1 log10 decline in HBsAG from baseline during the biweekly dosing.

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# CLAIMS

**WHAT IS CLAIMED IS:**

1. A method comprising detecting a hepatitis B virus (HBV) infected patient's genotype at rs12079860, wherein a C/C genotype at rs12079860 is indicative of a patient that has a higher likelihood of responding to an HBV antigen inhibitor as compared to an HBV infected patient having a different genotype at rs12079860.

2. A method comprising:

a) analyzing a biological sample obtained from a hepatitis B virus (HBV) infected patient to detect the patient's genotype at rs12079860; and

b) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860.

3. A method comprising:

a) obtaining a biological sample from a hepatitis B virus (HBV) infected patient; and

b) analyzing the sample to detect the patient's genotype at rs12079860; and

c) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860.

4. The method of any one of claims 1-3, further comprising administering an effective amount of an HBV antigen inhibitor to the HBV infected patient having a C/C genotype at rs12079860.

5. A method comprising:

a) obtaining a biological sample from a hepatitis B virus (HBV) infected patient;

b) analyzing the sample to detect the patient's genotype at rs12079860; and

c) administering an effective amount of an HBV antigen inhibitor to a patient having a C/C genotype at rs12079860.

6. A method comprising:

a) obtaining a biological sample from a hepatitis B virus (HBV) infected patient;

b) analyzing the sample to detect the patient's genotype at rs12079860;

c) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

d) administering an effective amount of an HBV antigen inhibitor to the patient.

7. A method of treating a hepatitis B virus (HBV) infected patient, comprising administering to the patient an effective amount of an HBV antigen inhibitor, wherein the patient had been determined to have a C/C genotype at rs12079860.

8. The method of any one of claims 4-7, wherein the HBV antigen inhibitor is a core antigen inhibitor.

9. The method of any one of claims 4-7, wherein the HBV antigen inhibitor is a surface antigen inhibitor.

10. The method of any one of claims 4-9, wherein the HBV antigen inhibitor is selected from an oligonucleotide, a small molecule or a polypeptide.

11. The method of claim 10, wherein the HBV antigen inhibitor is a small molecule.

12. The method of claim 10, wherein the HBV antigen inhibitor is an oligonucleotide.

13. The method of claim 12, wherein the oligonucleotide is a siRNA molecule.

14. The method of claim 13, wherein the HBV antigen inhibitor is an siRNA molecule selected from the siRNA molecules described in Tables A and B.

15. The method of claim 13, wherein the HBV antigen inhibitor is a composition comprising a combination of two or more siRNA molecules selected from the siRNA molecules described in Tables A and B.

16. The method of claim 13, wherein the HBV antigen inhibitor is a composition comprising a combination of three or more siRNA molecules selected from the siRNA molecules described in Tables A and B.

17. The method of claim 16, wherein the HBV antigen inhibitor is a composition comprising siRNA 67m (SEQ ID NO:142 and 143), siRNA 71m (SEQ ID NO:144 and 145) and siRNA 74m (SEQ ID NO:10 and 24).

18. A method comprising:

a) obtaining a biological sample from a hepatitis B virus (HBV) infected patient;

b) analyzing the sample to detect the patient's genotype at rs12079860;

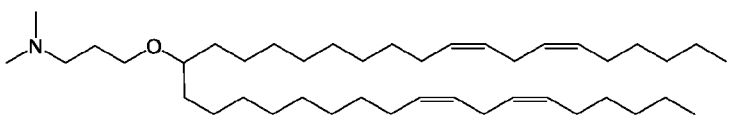
c) identifying the HBV infected patient as having a higher likelihood of responding to an HBV antigen inhibitor when a C/C genotype at rs12079860 is detected, as compared to a patient having a different genotype at rs12079860; and

d) administering an effective amount of an HBV antigen inhibitor to the patient, wherein the HBV antigen inhibitor is a composition comprising siRNA 67m (SEQ ID NO:142 and 143), siRNA 71m (SEQ ID NO:144 and 145) and siRNA 74m (SEQ ID NO:10 and 24).

19. The method of any one of claims 12-18, wherein the oligonucleotide (e.g., siRNA) is comprised in a lipid nanoparticle formulation, wherein the lipid nanoparticle formulation comprises a cationic lipid and a non-cationic lipid.

20. The method of claim 19, wherein the cationic lipid is selected from the group consisting of 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA; Compound (**515**)) , 3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (DLin-MP-DMA; Compound (**508**)), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) (Compound (**507**)), (6Z,16Z)-12-((Z)-dec-4-enyl)docosa-6,16-dien-11-yl 5-(dimethylamino)pentanoate (Compound (**513**)), a salt thereof, and a mixture thereof.

21. The method of claim 20, wherein the cationic lipid is:



3-((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yloxy)-N,N-dimethylpropan-1-amine (**508**).

22. The method of any one of claims 19-21, wherein the non-cationic lipid is cholesterol or a derivative thereof.

23. The method of any one of claims 19-21, wherein the non-cationic lipid is a phospholipid.

24. The method of any one of claims 19-21, wherein the non-cationic lipid is a mixture of a phospholipid and cholesterol or a derivative thereof.

25. The method of claim 23 or 24, wherein the phospholipid is selected from the group consisting of dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and a mixture thereof.

26. The method of claim 25, wherein the phospholipid is DSPC.

27. The method of any one of claims 19-26, wherein the lipid formulation further comprises a conjugated lipid that inhibits aggregation of particles.

28. The method of claim 27, wherein the conjugated lipid that inhibits aggregation of particles is a polyethyleneglycol (PEG)-lipid conjugate.

29. The method of claim 28, wherein the PEG-lipid conjugate is selected from the group consisting of a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, a PEG-phospholipid conjugate, a PEG-ceramide (PEG-Cer) conjugate, a PEG-dimyristyloxypropyl (PEG-DMA) conjugate and a mixture thereof.

30. The method of claim 29, wherein the PEG-lipid conjugate is a PEG-C-DMA conjugate.

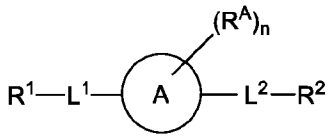
31. The method of any one of claims 19-30, wherein the cationic lipid comprises from about 48 mol % to about 62 mol % of the total lipid present in each particle within the formulation.

32. The method of any one of claims 24-31, comprising a phospholipid and cholesterol or cholesterol derivative, wherein the phospholipid comprises from about 7 mol % to about 17 mol % of the total lipid present in each particle within the formulation and the cholesterol or derivative thereof comprises from about 25 mol % to about 40 mol % of the total lipid present in each particle within the formulation.

33. The method of any one of claims 27-32, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 0.5 mol % to about 3 mol % of the total lipid present in each particle within the formulation.

34. The method of any one of claims 12-14, wherein the oligonucleotide (e.g., siRNA) is conjugated to a targeting moiety.

35. The method of claim 34, wherein the oligonucleotide (e.g., siRNA) is comprised within a compound of formula (I):




(I)

wherein:

$R^1$ a is targeting ligand;

$L^1$ is absent or a linking group;

$L^2$ is absent or a linking group;

$R^2$ is the oligonucleotide (e.g., siRNA);

the ring A is absent, a 3-20 membered cycloalkyl, a 5-20 membered aryl, a 5-20 membered heteroaryl, or a 3-20 membered heterocycloalkyl;

each $R^A$ is independently selected from the group consisting of hydrogen, hydroxy, CN, F, Cl, Br, I, -$C_{1-2}$ alkyl-$OR^B$, $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl; wherein the $C_{1-10}$ alkyl $C_{2-10}$ alkenyl, and $C_{2-10}$ alkynyl are optionally substituted with one or more groups independently selected from halo, hydroxy, and $C_{1-3}$ alkoxy;

$R^B$ is hydrogen, a protecting group, a covalent bond to a solid support, or a bond to a linking group that is bound to a solid support; and

n is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

or a salt thereof.

36. The method of any one of claims 4-35, wherein an effective amount of an HBV antigen inhibitor for a patient having a C/C genotype at rs12079860 is less than an effective amount of an HBV antigen inhibitor for a patient having a different genotype at rs12079860.

37. The method of any one of claims 4-35, wherein an HBV infected patient having a C/C genotype at rs12079860 is administered a different HBV antigen inhibitor treatment regimen than an HBV infected patient having a different genotype at rs12079860.

38. The method of claim 37, wherein the HBV infected patient having a C/C genotype at rs12079860 is administered a lower dosage of the HBV antigen inhibitor and/or is administered the HBV antigen inhibitor for a shorter period of time as compared to an HBV infected patient having a different genotype at rs12079860.

39. The method of any one of claims 4-38, further comprising administering at least one additional therapeutic agent.

40. The method of claim 39, wherein the at least one additional therapeutic agent selected from the group consisting of:

(A) an agent that controls viral replication;

(B) an agent that reduces viral Ags;

(C) an immune enhancer; and

(D) an immune stimulant.

41. The method of any one of claims 1-40, wherein the HBV infected patient is further infected with hepatitis D virus (HDV).

42. An HBV antigen inhibitor for the prophylactic or therapeutic treatment of a hepatitis B virus infection in a patient determined to have a C/C genotype at rs12079860.

43. The use of an HBV antigen inhibitor to prepare a medicament for treating a hepatitis B virus infection in a patient determined to have a C/C genotype at rs12079860.

44. A kit comprising:

a) an HBV antigen inhibitor;

b) instructions for administering the inhibitor to a hepatitis B virus (HBV) infected patient determined to have a C/C genotype at rs12079860.

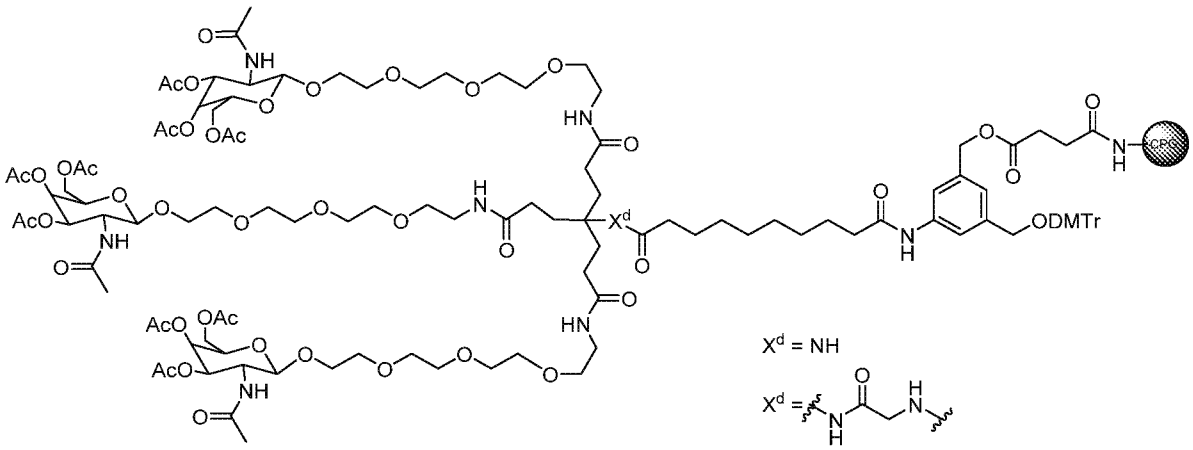



Figure 1

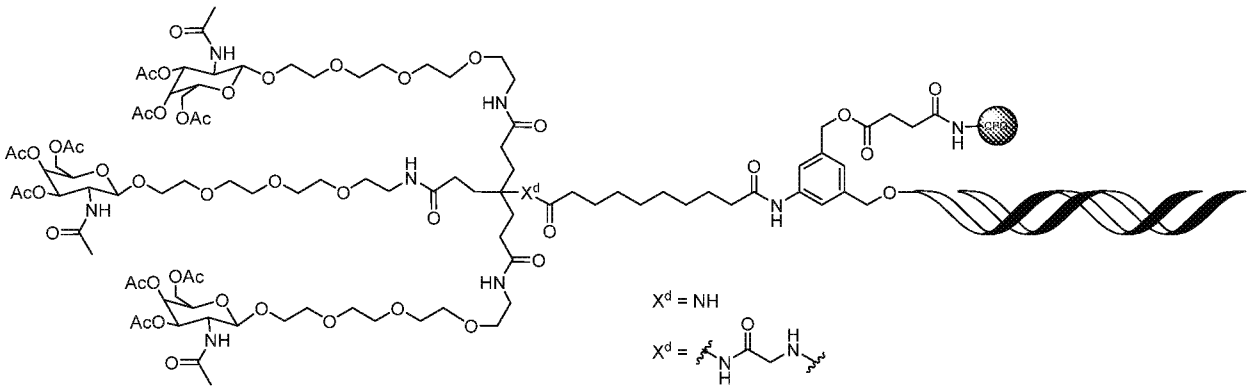



Figure 2

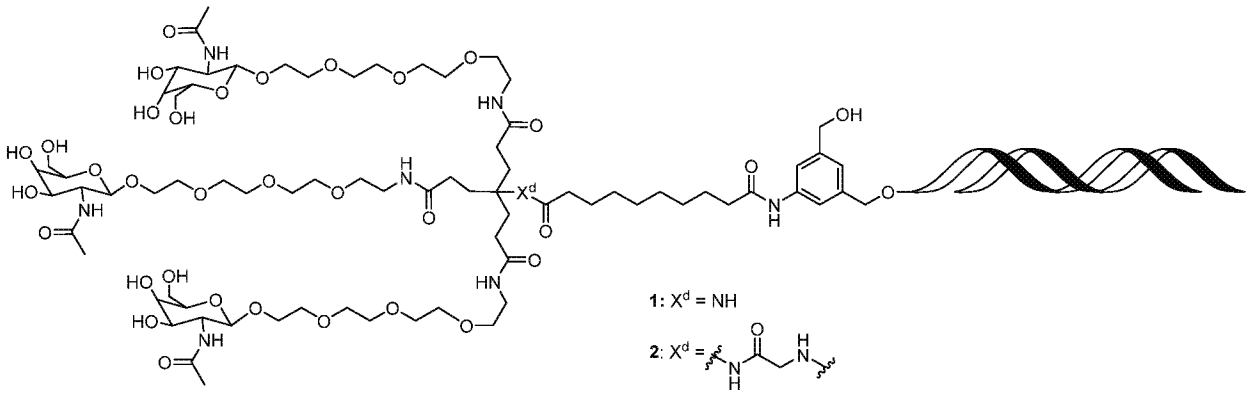



Figure 3