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(54) Title: CYCLIC-DISULFIDE MODIFIED PHOSPHATE BASED OLIGONUCLEOTIDE PRODRUGS

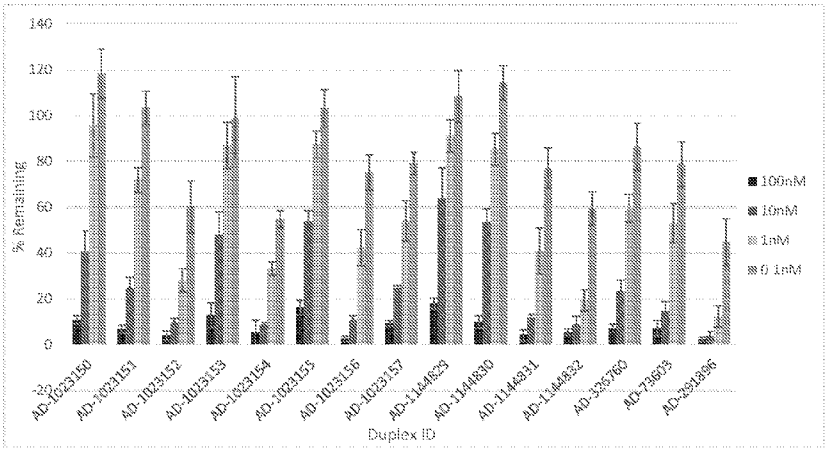
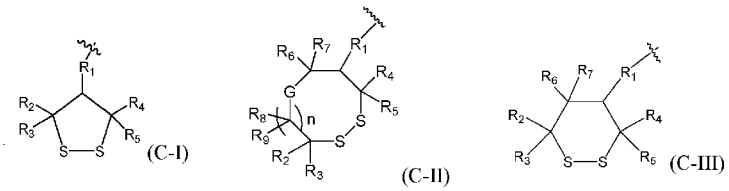


Figure 1



(57) Abstract: This invention relates to a compound comprising a structure of formula (I): cyclic disulfide moiety — phosphorus coupling group (I). The cyclic disulfide moiety has the structure of (C-I), (C-II), or (C-III). This invention also relates to an oligonucleotide comprising one or more compounds that comprise the structure of formula (I), wherein at least one phosphorus coupling group contains a nucleoside or oligonucleotide. The invention also relates to a pharmaceutical composition comprising the oligonucleotide described herein and a method of reducing or inhibiting the expression of a target gene by administering to the subject a therapeutically effective amount of the oligonucleotide described herein.

WO 2022/147214 A2

[Continued on next page]

NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

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Cyclic-Disulfide Modified Phosphate Based Oligonucleotide Prodrugs

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority to U.S. Provisional Application No. 63/132,535 filed December 31, 2020; and U.S. Provisional Application No. 63/287,833 filed December 9, 2021, both of which are herein incorporated by reference in their entirety.

FIELD OF INVENTION

[0002] This invention generally relates to the field of modified phosphate based oligonucleotide prodrugs.

BACKGROUND

[0003] Phosphate esters are important intermediates in formation of nucleotides and their assembly into RNA and DNA. Within the cell, the phosphate group commonly serves as a tunable leaving group. Phosphate esters are charged at a physiological pH, which serve to bind the phosphate esters to the active site of an enzyme. However, in order for the phosphate esters to bind the enzymes, they must first penetrate the membrane to access the enzyme, as charged molecules can have difficulty traversing the cell membrane other than by endocytosis. This limitation may be ameliorated in the case of compounds with larger, more lipophilic substituents.

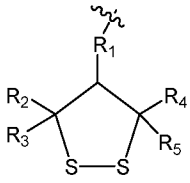
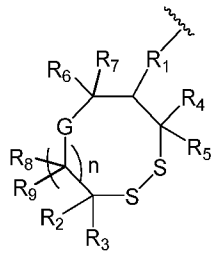
[0004] Alternatively, prodrug approaches have been researched to temporarily mask any negative charges of the phosphate esters on the oligonucleotide at a physiological pH. A prodrug is an agent that is administered in an inactive or significantly less active form, and that undergoes chemical or enzymatic transformations *in vivo* to yield the active parent drug under different stimuli. Prodrug approaches to mask the negative charges of phosphate groups of the oligonucleotide with cell-cleavable protecting/masking groups can offer a number of advantages over their non-protected counterparts including, *e.g.*, enhancing cell penetration and avoiding or minimizing degradation in serum via cellular sequestration.

[0005] However, the prodrug approach still has substantial challenges, partially because it is difficult to choose the best masking group. For instance, cellular cleavage of the protecting groups can often generate products which are viewed as disadvantageous or even toxic. Moreover, the protecting groups must strike a balance between allowing absorption in the

intestines and allowing cleavage in the blood or target cell.

[0006] Thus, there is a continuing need for developing new and improved modified phosphate prodrugs for masking internucleotide phosphate linkages of an oligonucleotide, in order to make effective and efficient oligonucleotide-based drugs, for efficient *in vivo* delivery and improved *in vivo* efficacy of oligonucleotides.

SUMMARY

[0007] One aspect of the invention relates to a compound comprising a structure of formula (I): cyclic disulfide moiety — phosphorus coupling group (I). The cyclic disulfide moiety has the structure of:  (C-I) or  (C-II). In these formulas:

R_1 is O or S, and is bonded to the P atom of the phosphorus coupling group;

R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

R_3 and R_5 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring;

G is O, N(R'), S, or $C(R^{14})(R^{15})$;

n is an integer of 0-6;

R^{13} is independently for each occurrence H, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, alkylcarbonyl, or arylcarbonyl, each of which can be optionally substituted with one or more R^{sub} groups;

R^{14}, R^{15}, and R^{16} are each independently H, halo, haloalkyl, alkyl, alkaryl, aryl, heteroaryl, aralkyl, hydroxy, alkyloxy, aryloxy, N(R')(R");

R' and R" are each independently H, alkyl, alkenyl, alkynyl, aryl, hydroxy, alkyloxy, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, or ω-hydroxy alkynyl, each of which

can be optionally substituted with one or more R^{sub} groups; and

R^{sub} is independently for each occurrence halo, haloalkyl, alkyl, alkaryl, aryl, aralkyl, hydroxy, alkyloxy, aryloxy, oxo, nitro, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, alkylamino, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, arylcarbonyl, acyloxy, cyano, or ureido.

[0008] In some embodiments, in the cyclic disulfide moiety:

R_1 is O;

G is CH_2;

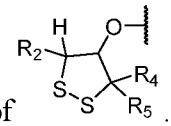
n is 0 or 1;

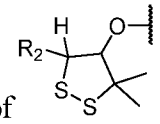
R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or C_1-C_6 alkylene-OR^{13}, N(R')(R") or C_1-C_6 alkylene-N(R')(R"), C_1-C_6 alkyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

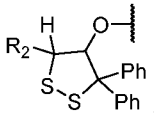
R_3 and R_5 are each independently H, halo, OR^{13} or C_1-C_6 alkylene-OR^{13}, N(R')(R") or C_1-C_6 alkylene-N(R')(R"), C_1-C_6 alkyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring of 6-8 atoms;

R^{13} is independently for each occurrence H, C_1-C_6 alkyl, aryl, alkylcarbonyl, or arylcarbonyl; and

R' and R" are each independently H or C_1-C_6 alkyl.

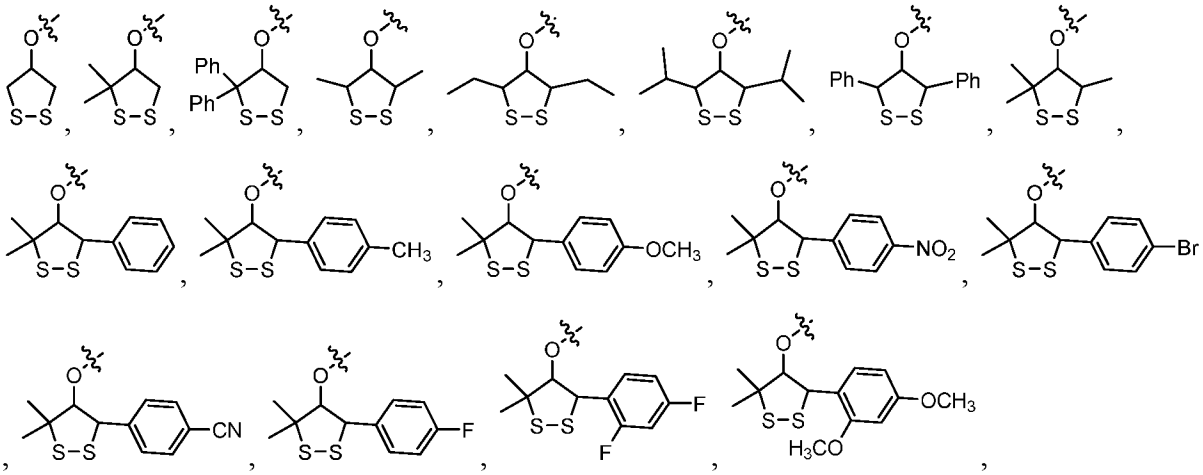
[0009] In some embodiments, the cyclic disulfide moiety has the structure of  . R_2 may be optionally substituted aryl, for instance, optionally substituted phenyl. In some embodiments, R_2 is mono-, di-, or tri-substituted phenyl. In one embodiment, R_2 is para-substituted phenyl. In some embodiments, R_2 is optionally substituted C_{1-6} alkyl. In one embodiment, R_2 is haloC_{1-6} alkyl. In one embodiment, R_2 is C_{1-6} alkyl.

[0010] In some embodiments, the cyclic disulfide moiety has the structure of 

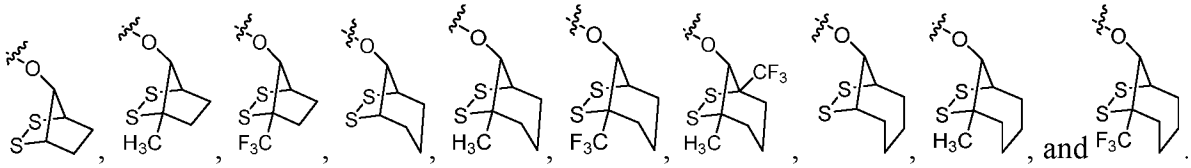
or  . R_2 may be optionally substituted aryl, for instance, optionally substituted phenyl. In some embodiments, R_2 is mono-, di-, or tri-substituted phenyl. In one

embodiment, R_2 is para-substituted phenyl. In some embodiments, R_2 is optionally substituted C_{1-6} alkyl. In one embodiment, R_2 is haloC_{1-6} alkyl. In one embodiment, R_2 is C_{1-6} alkyl.

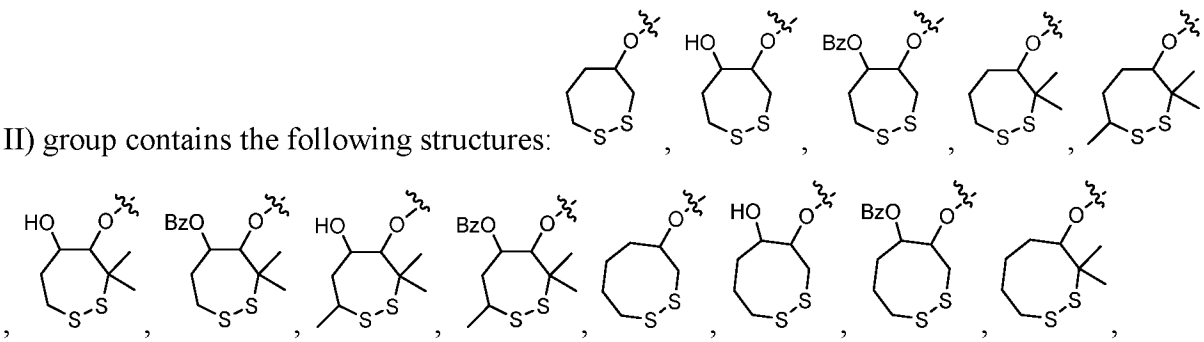
[0011] In some embodiments, the cyclic disulfide moiety has the structure selected from one of the following Ia), Ib), and II) groups. Ia) group contains the following structures:

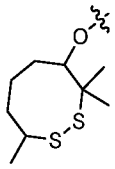
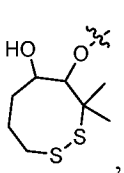
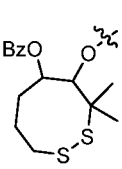
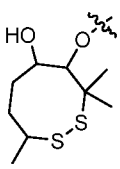
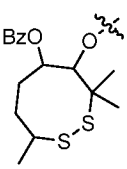


Ib) group contains the following structures:

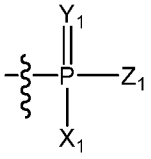
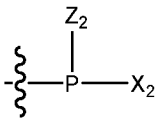


II) group contains the following structures:







, and  .

[0012] In some embodiments, the phosphorus coupling group has the structure of:


(P-I) or 
(P-II). In these formulas:

X_1 and Z_1 are each independently H, OH, OM, OR^{13}, SH, SM, SR^{13}, C(O)H, S(O)H, or alkyl, each of which can be optionally substituted with one or more R^{sub} groups, N(R')(R"), $B(R^{13})_3$, BH_3^-, Se; or D-Q, wherein D is independently for each occurrence absent, O, S, N(R'), alkylene, each of which can be optionally substituted with one or more R^{sub} groups, and Q is independently for each occurrence a nucleoside or oligonucleotide;

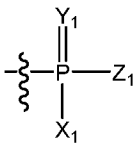
X_2 and Z_2 are each independently N(R')(R"), OR^{18}, or D-Q, wherein D is independently for each occurrence absent, O, S, N, N(R'), alkylene, each of which can be optionally substituted with one or more R^{sub} groups, and Q is independently for each occurrence a nucleoside or oligonucleotide,

Y_1 is S, O, or N(R');

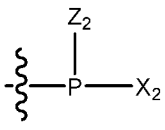
M is an organic or inorganic cation; and

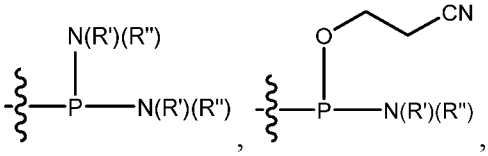
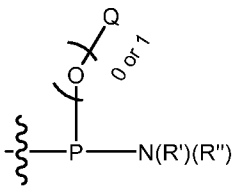
R^{18} is H or alkyl, optionally substituted with one or more R^{sub} groups.

[0013] In some embodiments, the phosphorus coupling group has the structure of

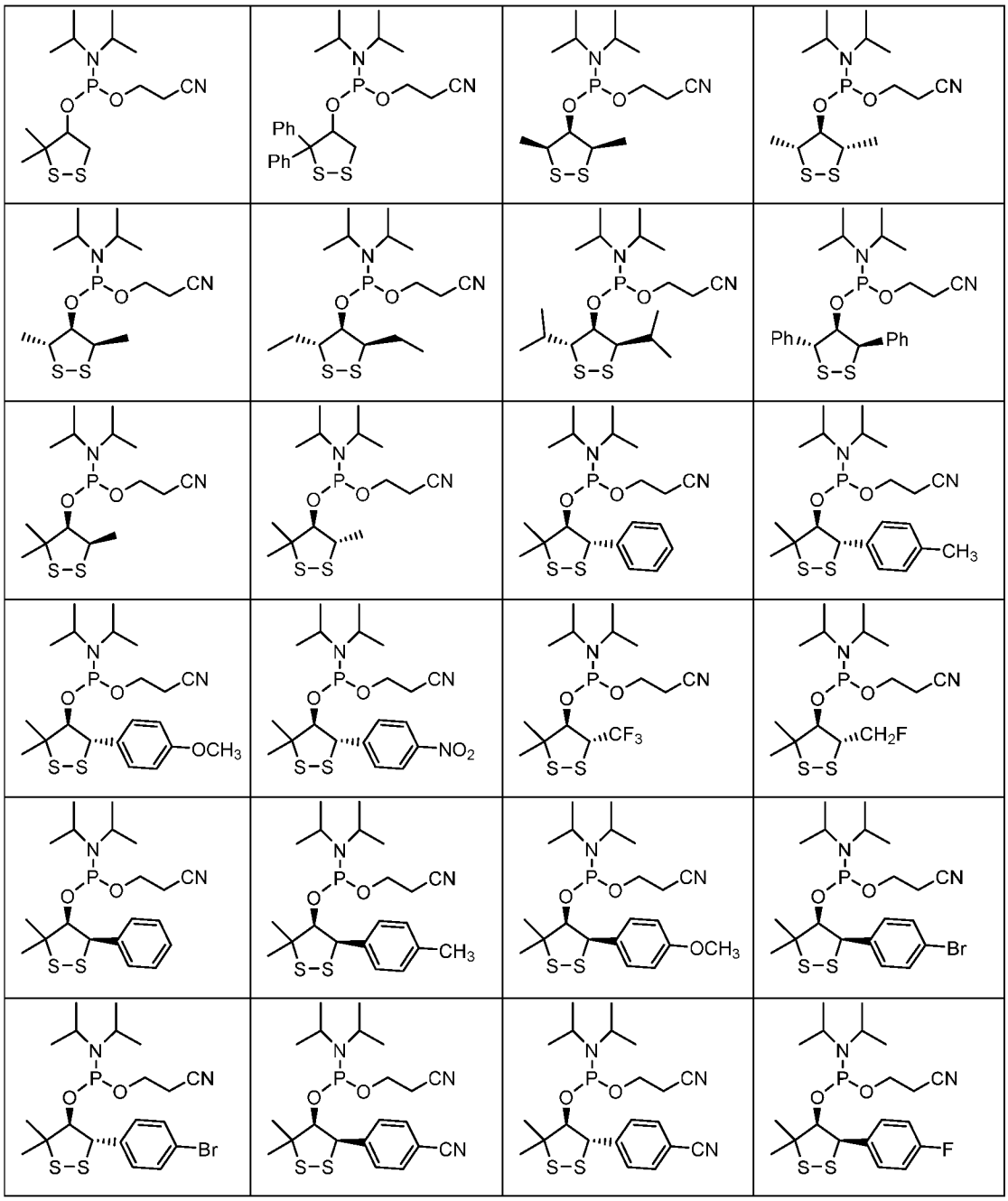

(P-I). In this formula: X_1 and Z_1 are each independently OH, OM, SH, SM, C(O)H, S(O)H, C_1-C_6 alkyl optionally substituted with one or more hydroxy or halo groups, or D-Q; D is independently for each occurrence absent, O, S, NH, C_1-C_6 alkylene optionally substituted with one or more halo groups; and Y_1 is S or O. In one embodiment, X_1 is OH or SH; and Z_1 is D-Q.

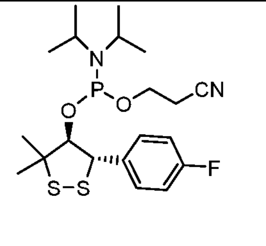
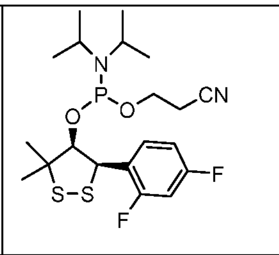
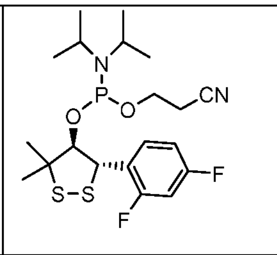
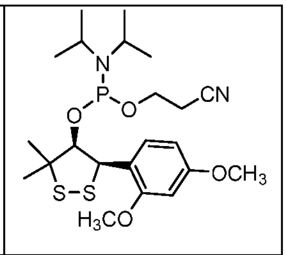
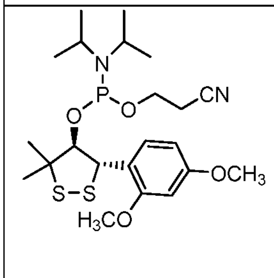
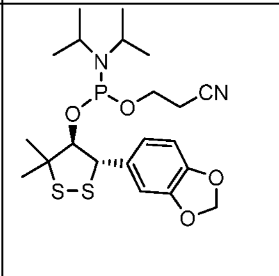
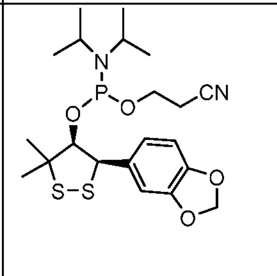
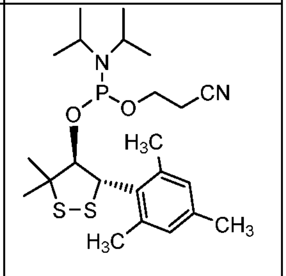
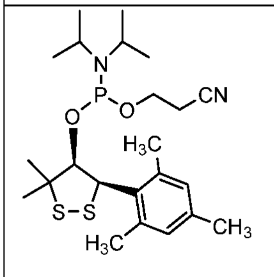
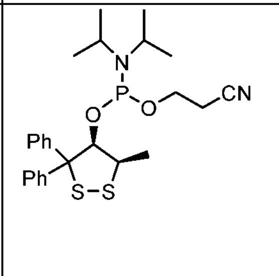
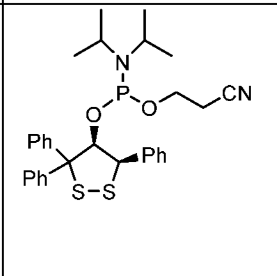
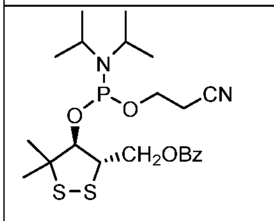
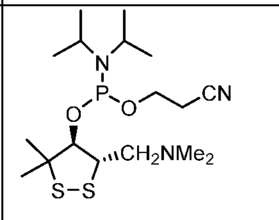
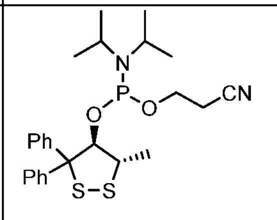
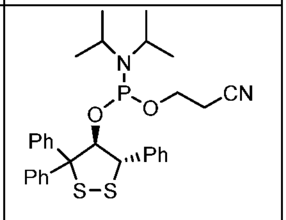
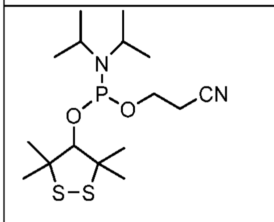
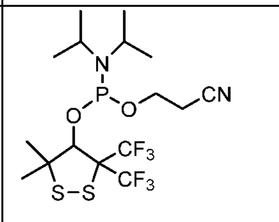
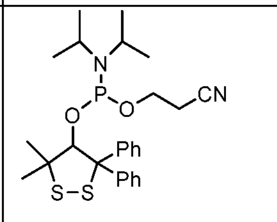
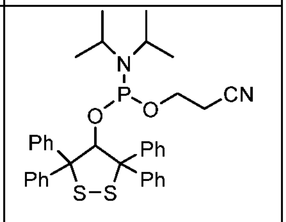
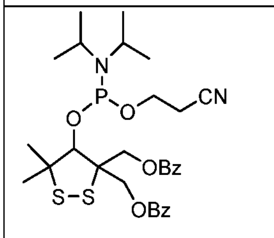
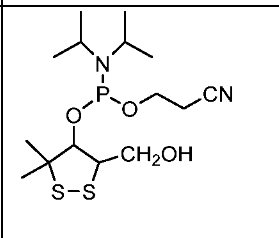
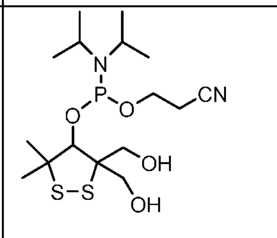
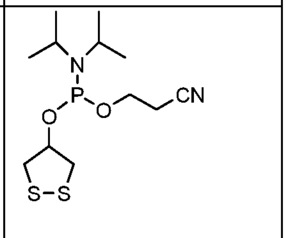
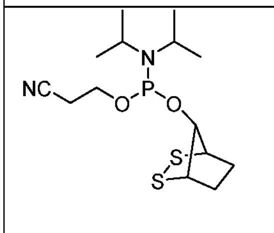
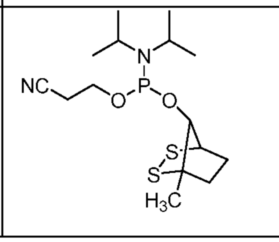
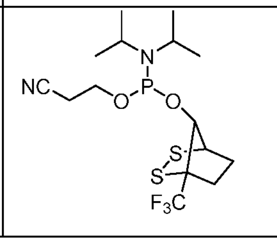
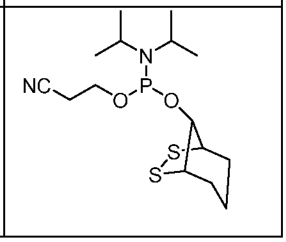
[0014] In some embodiments, the phosphorus coupling group has the structure of

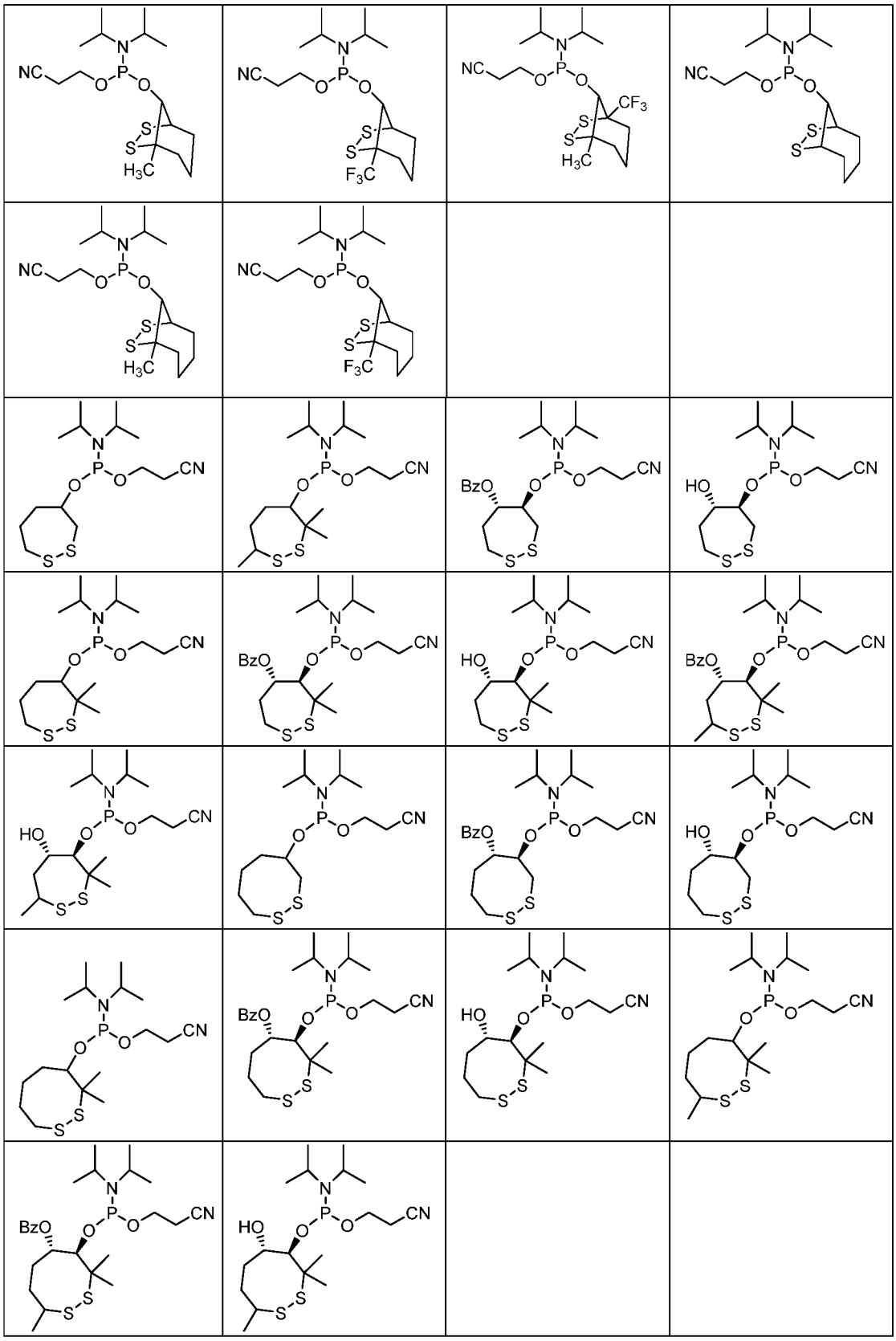

(P-II). In this formula, X_2 is N(R')(R"); Z_2 is X_2, OR^{18}, or D-Q; R^{18} is H or C_1-C_6 alkyl substituted with cyano; and R' and R'' are each independent C_1-C_6 alkyl.

[0015] In one embodiment, the phosphorus coupling group has a structure selected from the group consisting of  , , and  . The variables R', R'', and Q are defined as above in formulas P-I and P-II.

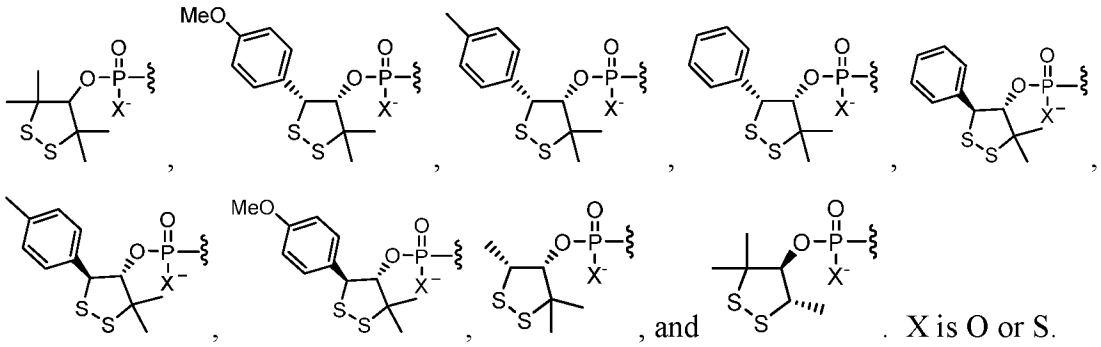
[0016] In one embodiment, the compound has a structure selected from one of the followings:





[0017] In one embodiment, the phosphorus coupling group has the structure of (P-I), and the cyclic disulfide moiety—$P(Y_1)(X_1)$- has a structure selected from the group consisting of:



, and . X is O or S.

[0018] In some embodiments, the compound contains one or more ligands connected to any one of R_2, R_3, R_4, R_5, R_6, R_7, R_8, and R_9 of the cyclic disulfide moiety, optionally via one or more linkers.

[0019] In some embodiments, the ligand is selected from the group consisting of an antibody, a ligand-binding portion of a receptor, a ligand for a receptor, an aptamer, a carbohydrate-based ligand, a fatty acid, a lipoprotein, folate, thyrotropin, melanotropin, surfactant protein A, mucin, glycosylated polyaminoacids, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipophilic moiety (e.g., a lipophilic moiety that enhances plasma protein binding), a cholesterol, a steroid, bile acid, vitamin B12, biotin, a fluorophore, and a peptide.

[0020] In certain embodiments, at least one ligand is a carbohydrate-based ligand targeting a liver tissue. In one embodiment, the carbohydrate-based ligand is selected from the group consisting of galactose, multivalent galactose, N-acetyl-galactosamine (GalNAc), multivalent GalNAc, mannose, multivalent mannose, lactose, multivalent lactose, N-acetyl-glucosamine (GlcNAc), multivalent GlcNAc, glucose, multivalent glucose, fucose, and multivalent fucose.

[0021] In certain embodiments, at least one ligand is a lipophilic moiety. In one embodiment, the lipophilicity of the lipophilic moiety, measured by $\log K_{ow}$, exceeds 0, or the hydrophobicity of the compound, measured by the unbound fraction in the plasma protein binding assay of the compound, exceeds 0.2.

[0022] In one embodiment, the lipophilic moiety contains a saturated or unsaturated C_4-C_{30} hydrocarbon chain, and an optional functional group selected from the group consisting of hydroxyl, amine, carboxylic acid, sulfonate, phosphate, thiol, azide, and alkyne. For instance, the lipophilic moiety contains a saturated or unsaturated C_6-C_{18} hydrocarbon chain.

[0023] In certain embodiments, at least one ligand targets a receptor which mediates delivery to a CNS tissue. In one embodiment, the ligand is selected from the group consisting of Angiopep-2, lipoprotein receptor related protein (LRP) ligand, bEnd.3 cell

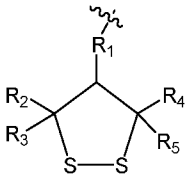
binding ligand, transferrin receptor (TfR) ligand, manose receptor ligand, glucose transporter protein, and LDL receptor ligand.

[0024] In certain embodiments, at least one ligand targets a receptor which mediates delivery to an ocular tissue. In one embodiment, the ligand is selected from the group consisting of trans-retinol, RGD peptide, LDL receptor ligand, and carbohydrate based ligands.

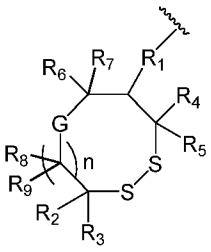
[0025] Another aspect of the invention relates to an oligonucleotide (e.g. a single-stranded iRNA agent or a double-stranded iRNA agent) comprising one or more structures of formula (I): cyclic disulfide moiety — phosphorus coupling group (I).

[0026] Another aspect of the invention relates to an oligonucleotide (e.g. a single-stranded iRNA agent or a double-stranded iRNA agent) comprising one or more structures of formula (II): cyclic disulfide moiety — P(Y)(X)-* (II).

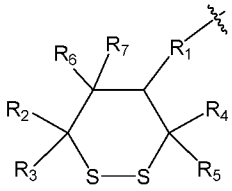
[0027] In both formulas (I) and (II), the cyclic disulfide moiety has the structure of:



(C-I),



(C-II), or



(C-III), or a salt thereof. In these formulas:

R_1 is O or S, and is bonded to the P atom of the phosphorus coupling group of formula (I), or the P atom of formula (II);

R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

R_3 and R_5 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring;

G is O, N(R'), S, or $C(R^{14})(R^{15})$;

n is an integer of 0-6;

R^{13} is independently for each occurrence H, alkyl, alkenyl, alkynyl, cycloalkyl, aryl,

aralkyl, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, alkylcarbonyl, or arylcarbonyl, each of which can be optionally substituted with one or more R^{sub} groups;

R^{14}, R^{15}, and R^{16} are each independently H, halo, haloalkyl, alkyl, alkaryl, aryl, heteroaryl, aralkyl, hydroxy, alkyloxy, aryloxy, N(R')(R");

R' and R" are each independently H, alkyl, alkenyl, alkynyl, aryl, hydroxy, alkyloxy, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, or ω-hydroxy alkynyl, each of which can be optionally substituted with one or more R^{sub} groups; and

R^{sub} is independently for each occurrence halo, haloalkyl, alkyl, alkaryl, aryl, aralkyl, hydroxy, alkyloxy, aryloxy, oxo, nitro, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, alkylamino, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, arylcarbonyl, acyloxy, cyano, or ureido;

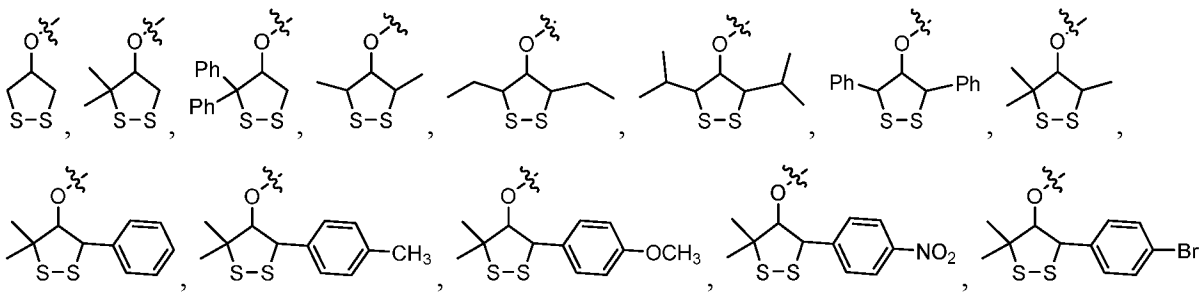
wherein, when the cyclic disulfide moiety has the structure of formula (C-III), at least one cyclic disulfide moiety is connected at the 5' end of the nucleoside or oligonucleotide.

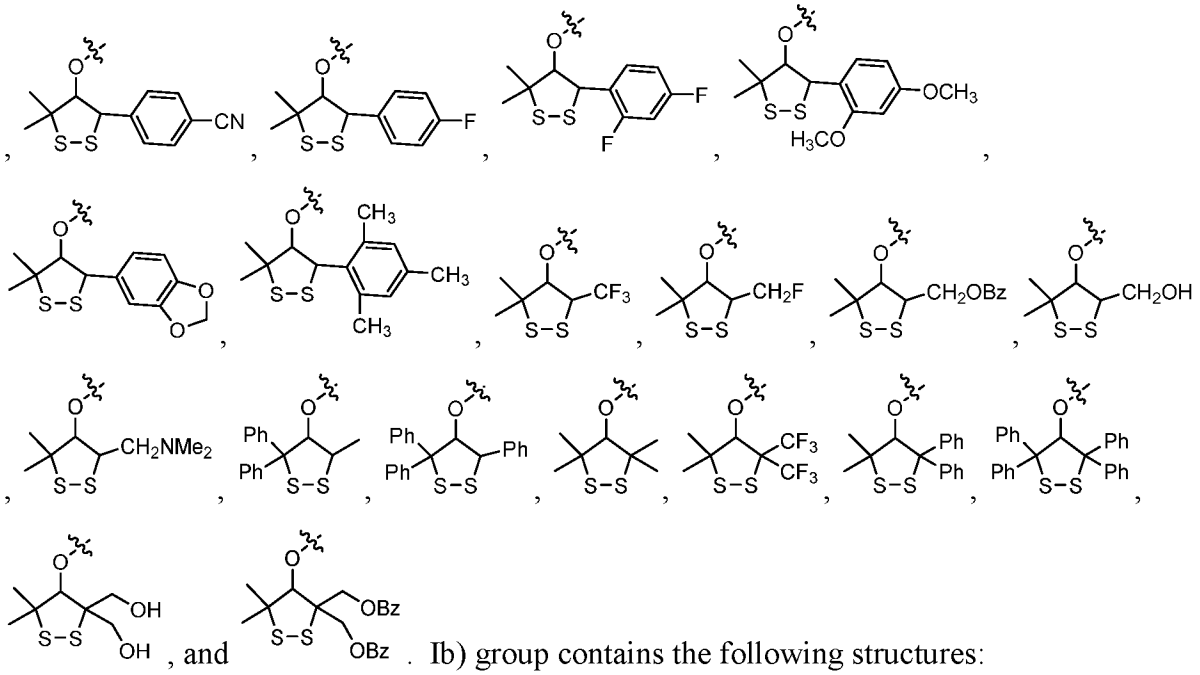
[0028] In Formula (I), at least one phosphorus coupling group contains a nucleoside or oligonucleotide.

[0029] In Formula (II), * represents the bond to the oligonucleotide, Y is absent, =O, or =S; X is OH, SH or X', wherein X' is OR^{13} or SR^{13}.

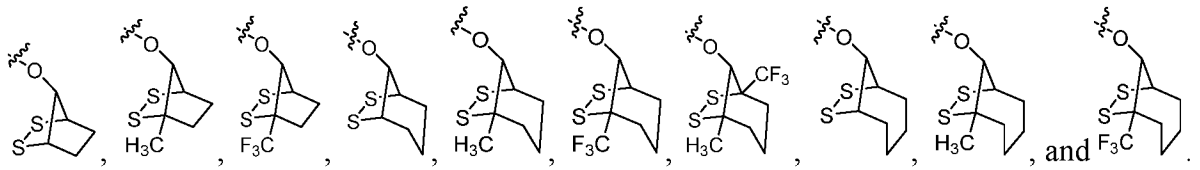
[0030] All the above embodiments relating to formula (C-I) and formula (C-II) of the cyclic disulfide moiety, all the formulas of the phosphorus coupling group, all the variables defined in these formulas, all the ligands, and all the subgenus and species structures relating to the compound, the cyclic disulfide moiety, and the phosphorus coupling group in the first aspect of the invention relating to the compound are suitable in these aspects of the invention relating to the oligonucleotide.

[0031] In some embodiments, the cyclic disulfide moiety has the structure selected from one of the following Ia), Ib), and II) groups. Ia) group contains the following structures:



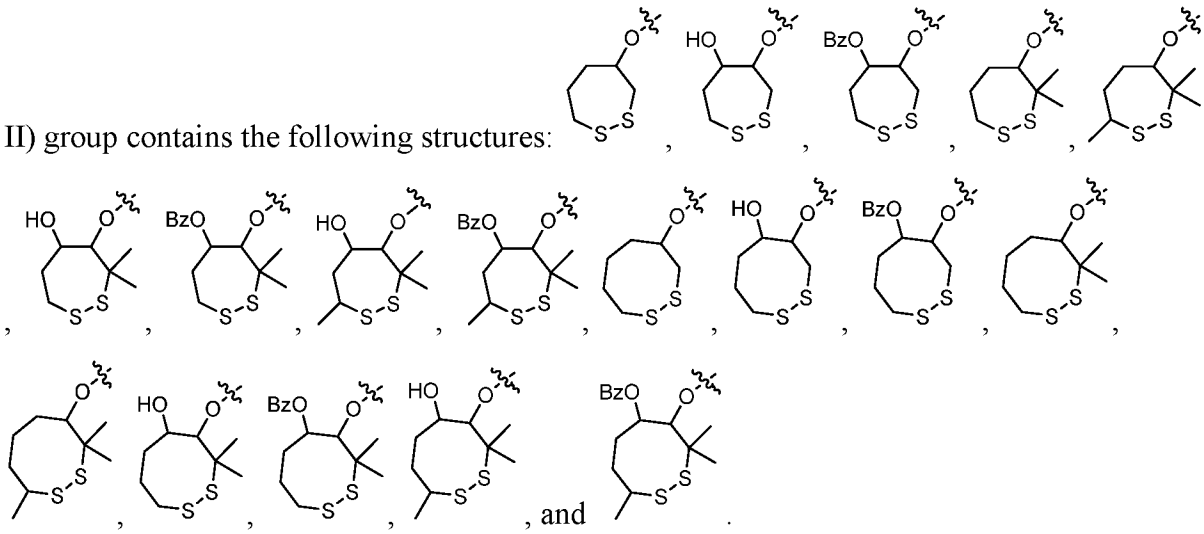


, and . Ib) group contains the following structures:



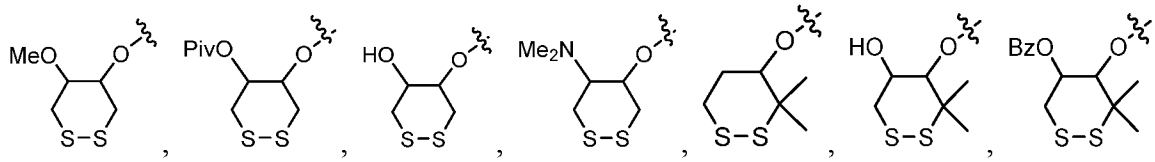
, and .

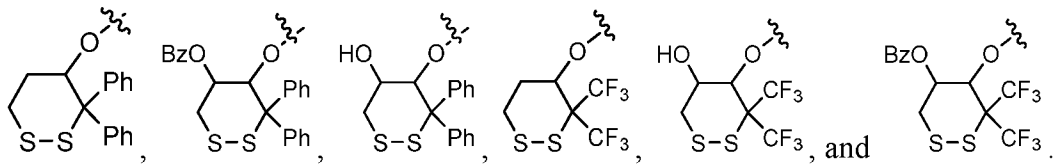
II) group contains the following structures:



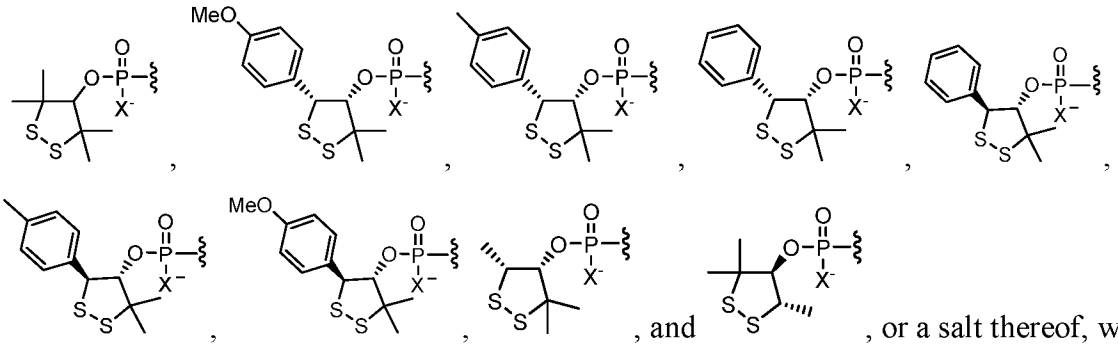
, and .

[0032] In some embodiments, the cyclic disulfide moiety has the structure selected from one of the structures from III) group. III) group contains the following structures:





[0033] In some embodiments, the oligonucleotide contains the structure selected from one of the following group consisting of:



, or a salt thereof, wherein X is O or S.

[0034] In some embodiments, the oligonucleotide contains a structure having the formula: cyclic disulfide moiety —P(O)(SH)-*, or a salt thereof.

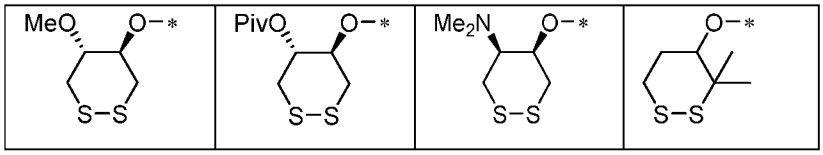
[0035] In some embodiments, the oligonucleotide contains a structure having the formula: cyclic disulfide moiety —P(O)(OH)-*, or a salt thereof.

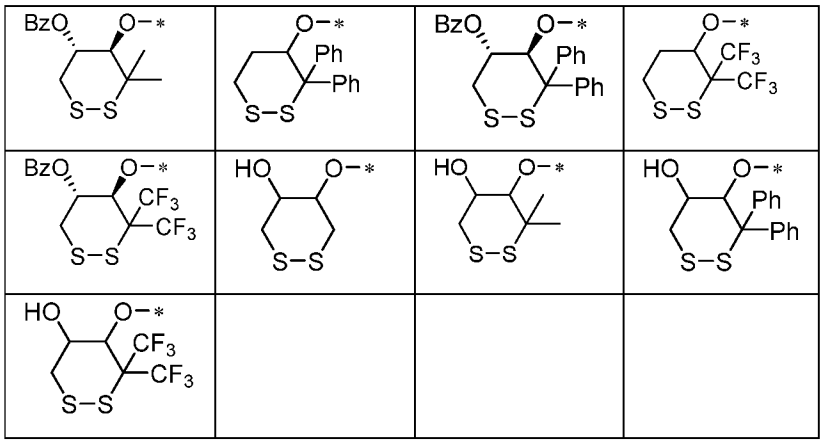
[0036] In some embodiments, the oligonucleotide contains a structure having the formula: cyclic disulfide moiety —P(O)(OR^{13})-* or a salt thereof. The variable R^{13} is as defined above.

[0037] In some embodiments, the oligonucleotide contains a structure having the formula: cyclic disulfide moiety —P(S)(OR^{13})-*, or a salt thereof. The variable R^{13} is as defined above.

[0038] In some embodiments, the oligonucleotide contains a structure having the formula: cyclic disulfide moiety —P(OR^{13})-*, or a salt thereof. The variable R^{13} is as defined above.

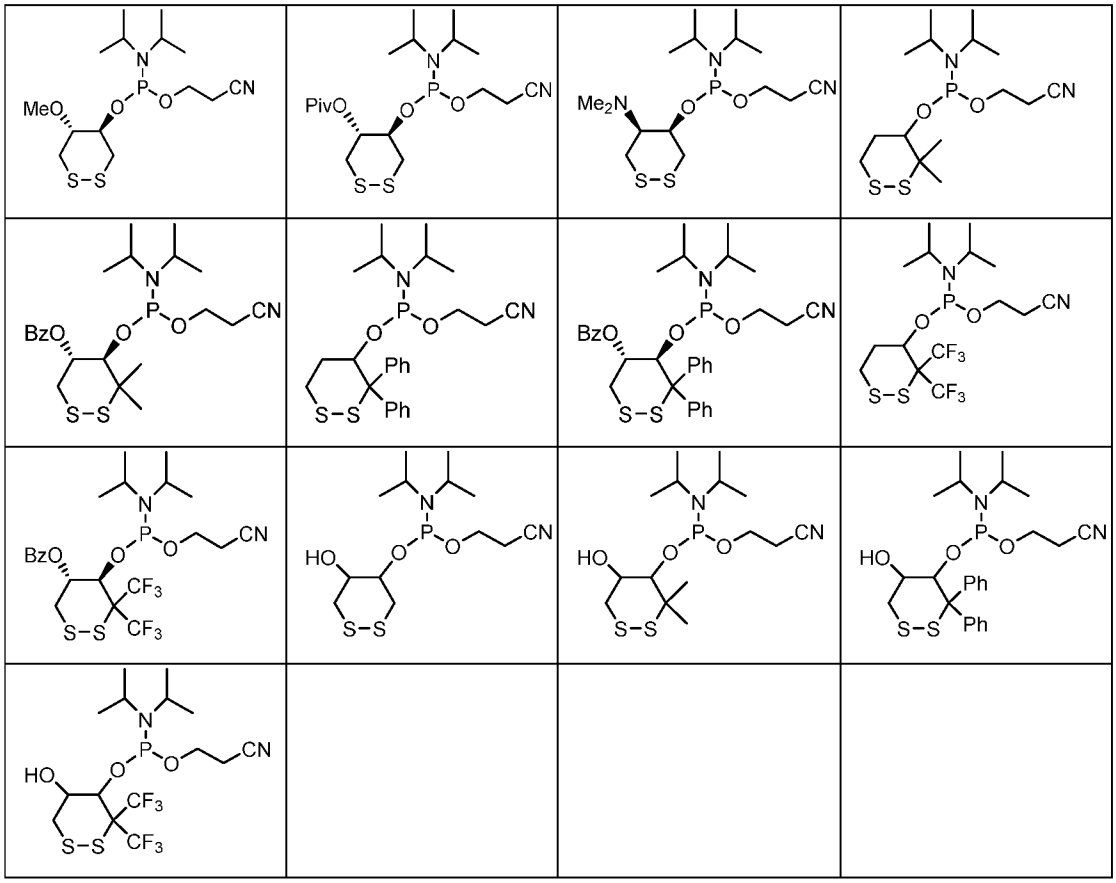
[0039] In some embodiments, the cyclic disulfide moiety has a structure selected from the group consisting of:

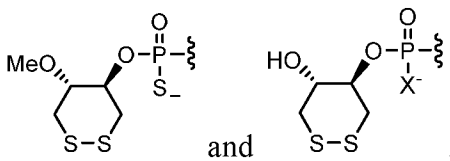




wherein * indicates the bond to the phosphorus atom of the -P(X)(Y)-* group.

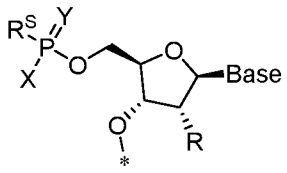
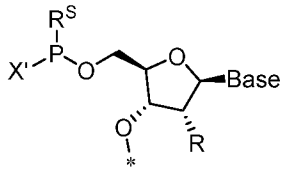
[0040] In some embodiments, the oligonucleotide contains a structure selected from one of the followings:



[0041] In one embodiment, the oligonucleotide contains a structure selected from the group consisting of  and . X is O or S.

[0042] In some embodiments, the oligonucleotide contains at least one cyclic disulfide

moiety at the 5'-end of the oligonucleotide.

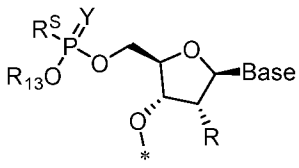
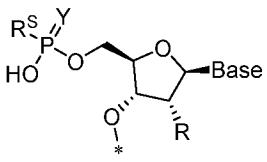
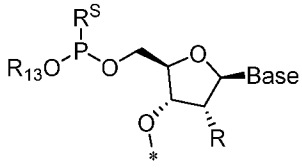
[0043] In some embodiments, the first nucleotide at the 5'-end of the oligonucleotide has the structure:  or  , or a salt thereof. In these structures:

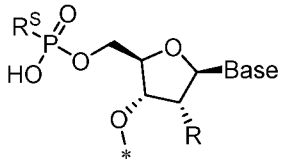
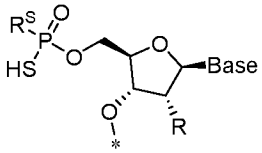
* represents a bond to the subsequent optionally modified internucleotide linkage;

Base is an optionally modified nucleobase;

R^S is the cyclic disulfide moiety; and

R is H, OH, O-methoxyalkyl, O-methyl, O-allyl, CH_2-allyl, fluoro, O-N-methylacetamido (O-NMA), O-dimethylaminoethoxyethyl (O-DMAEOE), O-aminopropyl (O-AP), or ara-F. The variables X, X', and Y, are defined as above in formula (II).

[0044] In some embodiments, the first nucleotide at the 5'-end of the oligonucleotide has the structure:  ,  or  , or a salt thereof. The variables Base, R^S, R^{13}, R, and Y are as defined above.

[0045] In some embodiments, the first nucleotide at the 5'-end of the oligonucleotide has the structure:  or  , or a salt thereof. The variables Base, R^S, and R are as defined above.

[0046] In some embodiments, Base in these structures is uridine. In some embodiments, R in these structures is methoxy. In some embodiments, R in these structures is hydrogen.

[0047] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at the 3'-end of the oligonucleotide.

[0048] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end of the oligonucleotide.

[0049] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end of the oligonucleotide, and at least one cyclic disulfide moiety at the 3'-end of the oligonucleotide.

[0050] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at an internal position of the oligonucleotide.

[0051] In some embodiments, the oligonucleotide is a single-stranded oligonucleotide.

[0052] In some embodiments, the oligonucleotide is a double-stranded oligonucleotide comprising a sense strand and an antisense strand.

[0053] In some embodiments, the sense and antisense strands are each 15 to 30 nucleotides in length. In one embodiment, the sense and antisense strands are each 19 to 25 nucleotides in length. In one embodiment, the sense and antisense strands are each 21 to 23 nucleotides in length.

[0054] In some embodiments, the oligonucleotide comprises a single-stranded overhang on at least one of the termini, e.g., 3' and/or 5' overhang(s) of 1-10 nucleotides in length, for instance, an overhang having 1, 2, 3, 4, 5, or 6 nucleotides in length. In some embodiments, both strands have at least one stretch of 1-5 (e.g., 1, 2, 3, 4, or 5) single-stranded nucleotides in the double stranded region. In one embodiment, the single-stranded overhang is 1, 2, or 3 nucleotides in length, optionally on at least one of the termini.

[0055] In some embodiments, the oligonucleotide may also have a blunt end, located at the 5'-end of the antisense strand (or the 3'-end of the sense strand), or vice versa. In one embodiment, the oligonucleotide comprises a 3' overhang at the 3'-end of the antisense strand, and optionally a blunt end at the 5'-end of the antisense strand. In one embodiment, the oligonucleotide has a 5' overhang at the 5'-end of the sense strand, and optionally a blunt end at the 5'-end of the antisense strand. In one embodiment, the oligonucleotide has two blunt ends at both ends of a double-stranded iRNA duplex.

[0056] In one embodiment, the sense strand of the oligonucleotide is 21-nucleotide in length, and the antisense strand is 23-nucleotide in length, wherein the strands form a double-stranded region of 21 consecutive base pairs having a 2-nucleotide long single-stranded overhangs at the 3'-end.

[0057] In one embodiment, the sense strand contains at least one cyclic disulfide moiety. In one embodiment, the antisense strand contains at least one cyclic disulfide moiety. In one embodiment, both the sense strand and the antisense strand each contain at least one cyclic disulfide moiety.

[0058] In one embodiment, the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end of the antisense strand and at least one targeting ligand at the 3'-end of the sense strand.

[0059] In some embodiments, the sense strand further comprises at least one phosphorothioate linkage at the 3'-end. In some embodiments, the sense strand comprises at least two phosphorothioate linkages at the 3'-end.

[0060] In some embodiments, the sense strand further comprises at least one phosphorothioate linkage at the 5'-end. In some embodiments, the sense strand comprises at least two phosphorothioate linkages at the 5'-end.

[0061] In some embodiments, the antisense strand further comprises at least one phosphorothioate linkage at the 3'-end. In some embodiments, the antisense strand comprises at least two phosphorothioate linkages at the 3'-end.

[0062] In some embodiments, the oligonucleotide further comprises a phosphate or phosphate mimic at the 5'-end of the antisense strand. In one embodiment, the phosphate mimic is a 5'-vinyl phosphonate (VP).

[0063] In some embodiments, the 5'-end of the antisense strand does not contain a 5'-vinyl phosphonate (VP).

[0064] In some embodiments, the oligonucleotide further comprises at least one terminal, chiral phosphorus atom.

[0065] A site specific, chiral modification to the internucleotide linkage may occur at the 5' end, 3' end, or both the 5' end and 3' end of a strand. This is being referred to herein as a "terminal" chiral modification. The terminal modification may occur at a 3' or 5' terminal position in a terminal region, *e.g.,* at a position on a terminal nucleotide or within the last 2, 3, 4, 5, 6, 7, 8, 9 or 10 nucleotides of a strand. A chiral modification may occur on the sense strand, antisense strand, or both the sense strand and antisense strand. Each of the chiral pure phosphorus atoms may be in either Rp configuration or Sp configuration, and combination thereof. More details regarding chiral modifications and chirally-modified dsRNA agents can be found in PCT/US18/67103, entitled "Chirally-Modified Double-Stranded RNA Agents," filed December 21, 2018, which is incorporated herein by reference in its entirety.

[0066] In some embodiments, the oligonucleotide further comprises a terminal, chiral modification occurring at the first internucleotide linkage at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration; a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration; and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp configuration or Sp configuration.

[0067] In one embodiment, the oligonucleotide further comprises a terminal, chiral modification occurring at the first and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration; a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration; and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

[0068] In one embodiment, the oligonucleotide further comprises a terminal, chiral modification occurring at the first, second, and third internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration; a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration; and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

[0069] In one embodiment, the oligonucleotide further comprises a terminal, chiral modification occurring at the first and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration; a terminal, chiral modification occurring at the third internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Rp configuration; a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration; and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

[0070] In one embodiment, the oligonucleotide further comprises a terminal, chiral modification occurring at the first and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration; a terminal, chiral modification occurring at the first, and second internucleotide linkages at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration; and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

[0071] In some embodiments, the oligonucleotide has at least two phosphorothioate internucleotide linkages at the first five nucleotides on the antisense strand (counting from the 5' end).

[0072] In some embodiments, the antisense strand comprises two blocks of one, two, or three phosphorothioate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 phosphate internucleotide linkages.

[0073] In some embodiments, the oligonucleotide contains one or more targeting ligands connected to any one of R_2, R_3, R_4, R_5, R_6, R_7, R_8, and R_9 of the cyclic disulfide moiety of the compound, optionally via one or more linkers.

[0074] In some embodiments, the targeting ligand is selected from the group consisting of an antibody, a ligand-binding portion of a receptor, a ligand for a receptor, an aptamer, a carbohydrate-based ligand, a fatty acid, a lipoprotein, folate, thyrotropin, melanotropin, surfactant protein A, mucin, glycosylated polyaminoacids, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipophilic moiety that enhances plasma protein binding, a cholesterol, a steroid, bile acid, vitamin B12, biotin, a fluorophore, and a peptide.

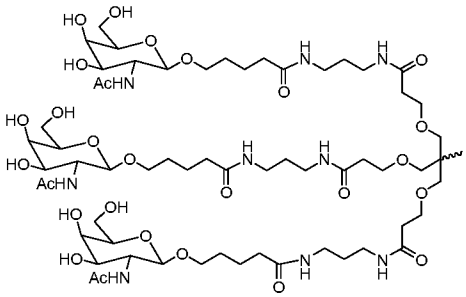
[0075] In some embodiments, at least one targeting ligand is a lipophilic moiety. In one embodiment, the lipophilicity of the lipophilic moiety, measured by $\log K_{ow}$, exceeds 0, or the hydrophobicity of the compound, measured by the unbound fraction in the plasma protein binding assay of the compound, exceeds 0.2. In one embodiment, the lipophilic moiety contains a saturated or unsaturated C_4-C_{30} hydrocarbon chain, and an optional functional group selected from the group consisting of hydroxyl, amine, carboxylic acid, sulfonate, phosphate, thiol, azide, and alkyne. For instance, the lipophilic moiety contains a saturated or unsaturated C_6-C_{18} hydrocarbon chain.

[0076] In some embodiments, at least one targeting ligand targets a receptor which mediates delivery to a specific CNS tissue. In one embodiment, the targeting ligand is selected from the group consisting of Angiopep-2, lipoprotein receptor related protein (LRP) ligand, bEnd.3 cell binding ligand, transferrin receptor (TfR) ligand, manose receptor ligand, glucose transporter protein, and LDL receptor ligand.

[0077] In some embodiments, at least one targeting ligand targets a receptor which mediates delivery to an ocular tissue. In one embodiment, the targeting ligand is selected from the group consisting of trans-retinol, RGD peptide, LDL receptor ligand, and carbohydrate-based ligands. In one embodiment, the targeting ligand is a RGD peptide, such as H-Gly-Arg-Gly-Asp-Ser-Pro-Lys-Cys-OH or Cyclo(-Arg-Gly-Asp-D-Phe-Cys).

[0078] In some embodiments, at least one targeting ligand targets a liver tissue. In some embodiments, the targeting ligand is a carbohydrate-based ligand. In one embodiment, the carbohydrate-based ligand is selected from the group consisting of galactose, multivalent

galactose, N-acetyl-galactosamine (GalNAc), multivalent GalNAc, mannose, multivalent mannose, lactose, multivalent lactose, N-acetyl-glucosamine (GlcNAc), multivalent GlcNAc, glucose, multivalent glucose, fucose, and multivalent fucose. In one embodiment, the targeting ligand is a GalNAc conjugate. For instance, the GalNAc conjugate is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker, such as:



[0079] In some embodiments, 100%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35% or 30% of the antisense and sense strand of the oligonucleotide is modified. For example, when 50% of the oligonucleotide is modified, 50% of all nucleotides present in the oligonucleotide contain a modification as described herein.

[0080] In some embodiments, the antisense and sense strands of the oligonucleotide comprise at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or virtually 100% 2'-O-methyl modified nucleotides.

[0081] In one embodiment, the oligonucleotide is a double-stranded dsRNA agent, and at least 50% of the nucleotides of the double-stranded dsRNA agent is independently modified with 2'-O-methyl, 2'-O-allyl, 2'-deoxy, or 2'-fluoro.

[0082] In one embodiment, the oligonucleotide is an antisense, and at least 50% of the nucleotides of the antisense is independently modified with LNA, CeNA, 2'-methoxyethyl, or 2'-deoxy.

[0083] In some embodiments, the sense and antisense strands comprise 12 or less, 10 or less, 8 or less, 6 or less, 4 or less, 2 or less, or no 2'-F modified nucleotides. In some embodiments, the oligonucleotide has 12 or less, 10 or less, 8 or less, 6 or less, 4 or less, 2 or less, or no 2'-F modifications on the sense strand. In some embodiments, the oligonucleotide has 12 or less, 10 or less, 8 or less, 6 or less, 4 or less, 2 or less, or no 2'-F modifications on the antisense strand. In one embodiment, the sense and the antisense strands comprise no more than ten 2'-fluoro modified nucleotides.

[0084] In some embodiments, the oligonucleotide contains one or more 2'-O modifications selected from the group consisting of 2'-deoxy, 2'-O-methoxyalkyl, 2'-O-methyl, 2'-O-allyl, 2'-C-allyl, 2'-fluoro, 2'-O-N-methylacetamido (2'-O-NMA), 2'-O-

dimethylaminoethoxyethyl (2'-O-DMAEOE), 2'-O-aminopropyl (2'-O-AP), and 2'-ara-F.

[0085] In some embodiments, the oligonucleotide contains one or more 2'-F modifications on any position of the sense strand or antisense strand.

[0086] In some embodiments, the oligonucleotide has less than 20%, less than 15%, less than 10%, less than 5% non-natural nucleotide, or substantially no non-natural nucleotide. Examples of non-natural nucleotide include acyclic nucleotides, LNA, HNA, CeNA, 2'-O-methoxyalkyl (*e.g.*, 2'-O-methoxymethyl, 2'-O-methoxyethyl, or 2'-O-2-methoxypropanyl), 2'-O-allyl, 2'-C-allyl, 2'-fluoro, 2'-O-N-methylacetamido (2'-O-NMA), a 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), 2'-O-aminopropyl (2'-O-AP), 2'-ara-F, L-nucleoside modification (such as 2'-modified L-nucleoside, e.g., 2'-deoxy-L-nucleoside), BNA abasic sugar, abasic cyclic and open-chain alkyl.

[0087] In some embodiments, the oligonucleotide has greater than 80%, greater than 85%, greater than 90%, greater than 95%, or virtually 100% natural nucleotides. For the purpose of these embodiments, natural nucleotides can include those having 2'-OH, 2'-deoxy, and 2'-OMe.

[0088] In some embodiments, the antisense strand contains at least one unlocked nucleic acids (UNA) or glycerol nucleic acid (GNA) modification, e.g., at the seed region of the antisense strand. In one embodiment, the seed region is at positions 2-8 (or positions 5-7) of the 5'-end of the antisense strand.

[0089] In one embodiment, the oligonucleotide comprises a sense strand and antisense strand each having a length of 15-30 nucleotides; at least two phosphorothioate internucleotide linkages at the first five nucleotides on the antisense strand (counting from the 5' end); wherein the duplex region is between 19 to 25 base pairs (preferably 19, 20, 21 or 22); wherein the oligonucleotide has less than 20%, less than 15%, less than 10%, less than 5% non-natural nucleotide, or substantially no non-natural nucleotide.

[0090] In one embodiment, the oligonucleotide comprises a sense strand and antisense strand each having a length of 15-30 nucleotides; at least two phosphorothioate internucleotide linkages at the first five nucleotides on the antisense strand (counting from the 5' end); wherein the duplex region is between 19 to 25 base pairs (preferably 19, 20, 21 or 22); wherein the oligonucleotide has greater than 80%, greater than 85%, greater than 95%, or virtually 100% natural nucleotides, such as those having 2'-OH, 2'-deoxy, or 2'-OMe.

[0091] One aspect of the invention provides an oligonucleotide comprising a sense strand and an antisense strand, each strand independently having a length of 15 to 35 nucleotides; at

least two phosphorothioate internucleotide linkages between the first five nucleotides counting from the 5' end of the antisense strand; at least three, four, five, or six 2'-deoxy modifications on the sense and/or antisense strands; wherein the oligonucleotide has a double stranded (duplex) region of between 19 to 25 base pairs; wherein the oligonucleotide comprises a ligand.

[0092] In one embodiment, the sense strand does not comprise a glycol nucleic acid (GNA).

[0093] It is understood that the antisense strand has sufficient complementarity to a target sequence to mediate RNA interference. In other words, the oligonucleotide is capable of inhibiting the expression of a target gene.

[0094] In one embodiment, the oligonucleotide comprises at least three 2'-deoxy modifications. The 2'-deoxy modifications are at positions 2 and 14 of the antisense strand, counting from 5'-end of the antisense strand, and at position 11 of the sense strand, counting from 5'-end of the sense strand.

[0095] In one embodiment, the oligonucleotide comprises at least five 2'-deoxy modifications. The 2'-deoxy modifications are at positions 2, 12 and 14 of the antisense strand, counting from 5'-end of the antisense strand, and at positions 9 and 11 of the sense strand, counting from 5'-end of the sense strand.

[0096] In one embodiment, the oligonucleotide comprises at least seven 2'-deoxy modifications. The 2'-deoxy modifications are at positions 2, 5, 7, 12 and 14 of the antisense strand, counting from 5'-end of the antisense strand, and at positions 9 and 11 of the sense strand, counting from 5'-end of the sense strand.

[0097] In one embodiment, the antisense strand comprises at least five 2'-deoxy modifications at positions 2, 5, 7, 12 and 14, counting from 5'-end of the antisense strand. The antisense strand has a length of 18-25 nucleotides, or a length of 18-23 nucleotides.

[0098] In one embodiment, the oligonucleotide comprises less than 20%, e.g., less than 15%, less than 10%, or less than 5% non-natural nucleotides, or comprises no non-natural nucleotides.

[0099] In one embodiment, the sense strand does not comprise a glycol nucleic acid (GNA); and wherein the oligonucleotide comprises less than 20%, e.g., less than 15%, less than 10%, or less than 5% non-natural nucleotides or comprises all natural nucleotides.

[0100] In one embodiment, at least one the sense and antisense strands comprises at least one, e.g., at least two, at least three, at least four, at least five, at least six, or at least seven or

more, 2'-deoxy modifications in a central region of the sense or antisense strand.

[0101] In one embodiment, the sense strand and/or the antisense strand comprises at least one, e.g., at least two, at least three, at least four, at least five, at least six, or at least seven or more, 2'-deoxy modifications in a central region of the sense strand and/or the antisense strand.

[0102] In some embodiment, the sense strand has a length of 18 to 30 nucleotides and comprises at least two 2'-deoxy modifications in the central region of the sense strand. For example, the sense strand has a length of 18 to 30 nucleotides and comprises at least two 2'-deoxy modifications within positions 7, 8, 9, 10, 11, 12, and 13, counting from 5'-end of the sense strand.

[0103] In one embodiment, the antisense strand has a length of 18 to 30 nucleotides and comprises at least two 2'-deoxy modifications in the central region of the antisense strand. For example, the antisense strand has length of 18 to 30 nucleotides and comprises at least two 2'-deoxy modifications within positions 10, 11, 12, 13, 14, 15 and 16, counting from 5'-end of the antisense strand.

[0104] In one embodiment, the oligonucleotide comprises a sense strand and an antisense strand; wherein the sense strand has a length of 17-30 nucleotide and comprises at least one 2'-deoxy modification in the central region of the sense strand; and wherein the antisense strand independently has a length of 17-30 nucleotides and comprises at least two 2'-deoxy modifications in the central region of the antisense strand.

[0105] In one embodiment, the oligonucleotide comprises a sense strand and an antisense strand; wherein the sense strand has a length of 17-30 nucleotide and comprises at least two 2'-deoxy modifications in the central region of the sense strand; and wherein the antisense strand independently has a length of 17-30 nucleotides and comprises at least one 2'-deoxy modification in the central region of the antisense strand.

[0106] In one embodiment, the sense strand comprises at least one, e.g., at least two, at least three, at least four, at least five, at least six, at least seven or more, 2'-deoxy modifications in a central region of the sense strand.

[0107] In one embodiment, the antisense strand comprises at least one, e.g., at least two, at least three, at least four, at least five, at least six, at least seven or more, 2'-deoxy modifications in a central region of the antisense strand.

[0108] In one embodiment, the oligonucleotide comprises less than 20%, e.g., less than 15%, less than 10%, or less than 5% non-natural nucleotides or the oligonucleotide comprises

all natural nucleotides; and wherein the sense strand and/or the antisense strand comprises at least one, e.g., at least two, at least three, at least four, at least five, at least six, at least seven or more, 2'-deoxy modifications in a central region of the sense strand and/or the antisense strand.

[0109] In one embodiment, the oligonucleotide comprises less than 20%, e.g., less than 15%, less than 10%, or less than 5% non-natural nucleotides or the oligonucleotide comprises all natural nucleotides; and wherein the sense strand comprises at least one, e.g., at least two, at least three, at least four, at least five, at least six, at least seven or more, 2'-deoxy modifications in a central region of the sense strand.

[0110] In one embodiment, the oligonucleotide comprises less than 20%, e.g., less than 15%, less than 10%, or less than 5% non-natural nucleotides or the oligonucleotide comprises all natural nucleotides; and wherein the antisense strand comprises at least one, e.g., at least two, at least three, at least four, at least five, at least six, at least seven or more, 2'-deoxy modifications in a central region of the antisense strand.

[0111] In one embodiment, when the oligonucleotide comprises less than 8 non-2'OMe nucleotides, the antisense stand comprises at least one DNA. For example, in any one of the embodiments of the invention when the oligonucleotide comprises less than 8 non-2'OMe nucleotides, the antisense stand comprises at least one DNA.

[0112] In one embodiment, when the antisense comprises two deoxy nucleotides and said nucleotides are at positions 2 and 14, counting from the 5'-end of the antisense strand, the oligonucleotide comprises 8 or less (e.g., 8, 7, 6, 5, 4, 3, 2, 1 or 0) non-2'OMe nucleotides. For example, in any one of the embodiments of the invention when the antisense comprises two deoxy nucleotides and said nucleotides are at positions 2 and 14, counting from the 5'-end of the antisense strand, the oligonucleotide comprises 0, 1, 2, 3, 4, 5, 6, 7 or 8 non 2'-OMe nucleotides.

[0113] Another aspect of the invention relates to a pharmaceutical composition comprising the oligonucleotide described herein, and a pharmaceutically acceptable excipient.

[0114] All the above embodiments relating to the oligonucleotide in the above aspect of the invention relating to the oligonucleotide are suitable in this aspect of the invention relating to the pharmaceutical composition.

[0115] In another aspect, the invention further provides a method for delivering the oligonucleotide of the invention to a specific target in a subject by subcutaneous or

intravenous administration. The invention further provides the oligonucleotide of the invention for use in a method for delivering said agents to a specific target in a subject by subcutaneous or intravenous administration.

[0116] Another aspect of the invention relates to a method of reducing or inhibiting the expression of a target gene in a subject, comprising administering to the subject the oligonucleotide described herein above in an amount sufficient to inhibit expression of the target gene.

[0117] All the above embodiments relating to the oligonucleotide in the above aspect of the invention relating to the oligonucleotide are suitable in this aspect of the invention relating to a method of reducing the expression of a target gene in a subject.

[0118] Another aspect of the invention relates to a method for modifying an oligonucleotide comprising contacting the oligonucleotide with the compound described herein above under conditions suitable for reacting the compound with the oligonucleotide, wherein the oligonucleotide comprises a free hydroxyl group.

[0119] In some embodiments, the free hydroxyl group is part of the 5'-terminal nucleotide. In some embodiments, the free hydroxyl group is part of the 3'-terminal nucleotide.

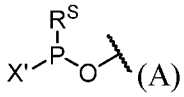
[0120] In some embodiments, the oligonucleotide comprises a 5'-OH group. In some embodiments, the oligonucleotide comprises a 3'-OH group.

[0121] In some embodiments, the conditions suitable for reacting the compound with the oligonucleotide comprise an acidic catalyst. For instance, the acid catalyst may be a substituted tetrazole. Suitable acidic catalysts include, but not limited to, 1H-tetrazole, 5-ethylthio-1H-tetrazole, 2-benzylthiotetrazole, 4,5-dicyanoimidazole, 5-nitrophenyl-1H-tetrazole, 5-(bis-3,5-trifluoromethylphenyl)-1H-tetrazole, 5-benzylthio-1H-tetrazole, 5-mthylthio-1H-tetrazole, 1-hydroxyl benzotriazole, 1-hydroxy-6-trifluoromethyl benzotriazole, 4-nitro-1-hydroxy-6-trifluoromethyl benzotriazole, pyridinium chloride, pyridinium bromide, pyridinium 4-methylbenzenesulfonate, 2,6-di(tert-butyl)pyridinium chloride, pyridinium trifluoroacetate, N-(phenyl)imidazolium triflate (N-PhIMT), N-(phenyl)-imidazolium perchlorate (N-PhIMP), N-(methyl)benzimidazolium triflate (NMeBIT), N-(p-acetylphenyl)imidazolium triflate (N-AcPhIMT), N-(phenyl)imidazolium tetrafluoroborate (N-PhIMTFB), imidazolium perchlorate (IMP), 4-(phenyl)-imidazolium triflate (4-PhIMT), benzimidazolium tetrafluoroborate (BITFB), imidazolium tetrafluoroborate (IMTFB), imidazolium triflate (IMT), benzimidazolium triflate (BIT), 2-

(phenyl)imidazolium triflate (2-PhIMT), N- (methyl)imidazolium triflate (N-MeIMT), 4-(methyl)imidazolium triflate (4-MeIMT), saccharin-1-methylimidazole, N-(cyanomethyl)pyrrolidinium triflate, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), dichloroacetic acid (DCA), and 2,4-dinitrobenzoic acids (2,4-DNBA), iron chloride (FeCl3), aluminum chloride (AlCl3), trifluoroboron etherate (BF3-OEt2), zirconium(IV) chloride (ZrCl4), and bismuth(III) chloride (BiCl3), trimethylchlorosilane, 2,4-dinitrophenol, 1-methyl-5-mercapto-tetrazole, and 1-phenyl-5-mercaptotetrazole.

[0122] All the above embodiments relating to the compound and the oligonucleotide in the above aspects of the invention are suitable in this aspect of the invention relating to a method for modifying an oligonucleotide.

[0123] Another aspect of the invention relates to a method for preparing a modified oligonucleotide, comprising: oxidizing a first oligonucleotide comprising a group of formula (A):

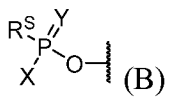

(A)

or a salt thereof, wherein:

R^S is a cyclic disulfide moiety;

X' is $-OR^{13}$ or $-SR^{13}$, wherein R^{13} is alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, alkylcarbonyl, or arylcarbonyl, each of which can be optionally substituted with one or more R^{sub} groups;

under conditions suitable for forming a modified oligonucleotide comprising a group of formula (B):

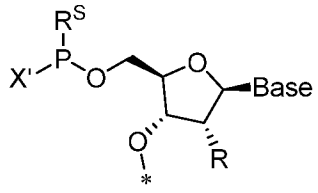

(B)

or a salt thereof, wherein Y is O or S; and X is -OH, -SH or X'. The variables Base, R^S, X, X', and Y are as defined above.

[0124] In some embodiments, the first nucleotide at the 5'-end of the first oligonucleotide comprises the group of formula (A) and the first nucleotide at the 5'-end of the modified oligonucleotide comprises the group of formula (B). In some mebodiments, the last nucleotide at the 3'-end of the first oligonucleotide comprises the group of formula (A) and the last nucleotide at the 3'-end of the modified oligonucleotide comprises the group of formula (B).

[0125] In some embodiments, the first nucleotide at the 5'-end of the first oligonucleotide

is according to formula (C):



(C)

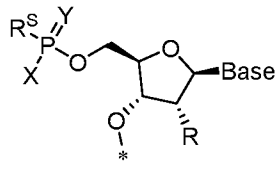
or a salt thereof, wherein:

* represents a bond to the subsequent optionally modified internucleotide linkage;

Base is an optionally modified nucleobase; and

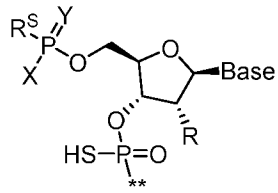
R is H, OH, O-methoxyalkyl, O-methyl, O-allyl, CH_2-allyl, fluoro, O-N-methylacetamido (O-NMA), O-dimethylaminoethoxyethyl (O-DMAEOE), O-aminopropyl (O-AP), or ara-F. The variables R^S and X' are as defined above.

[0126] In some embodiments, the first nucleotide at the 5'-end of the modified oligonucleotide has the structure of formula (D):

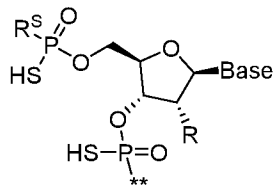


(D). The variables Base, R, R^S, X, and Y are as defined above.

[0127] In some embodiments, the first nucleotide at the 5'-end of the modified oligonucleotide has the structure of formula (E) or (F):



(E),



(F),

or a salt thereof, wherein ** represents the bond to the subsequent nucleotide. The variables Base, R, R^S, X, and Y are as defined above.

[0128] In some embodiments, the conditions suitable for forming a modified oligonucleotide comprise using an oxidizing agent selected from the group consisting of iodine; sulfur; a peroxide; a peracid; phenylacetyl disulfide; 3H-1,2-benzodithiol-3-one 1,1-dioxide; dixanthogen; 5-ethoxy-3H-1,2,4-dithiazol-3-one; 3-[(dimethylaminomethylene)amino]-3H-1,2,4-dithiazole-5-thione (DDTT); dimethyl

sulfoxide; and N-bromosuccinimide. For instance, the oxidizing agents may be a peracid (e.g., m-chloroperbenzoic acid), or a peroxide (e.g., tert-butyl hydroperoxide or trimethylsilyl peroxide).

[0129] All the above embodiments relating to the compound and the oligonucleotide in the above aspects of the invention are suitable in this aspect of the invention relating to a method for preparing a modified oligonucleotide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0130] **Figure 1** is a graph depicting *in vitro* activity of F12 siRNAs containing the modified phosphate prodrugs at the 5' end in primary mouse hepatocytes, after transfection with RNAiMAX at 0.1, 1, 10, and 100 nm concentrations and analyzed 24 hours post-transfection. Percentage of F12 message remaining was determined by qPCR and were plotted against the control.

[0131] **Figure 2** is a graph depicting *in vitro* activity of F12 siRNA duplexes containing the modified phosphate prodrugs at the 5' end in primary mouse hepatocytes after incubating at 0.1, 1, 10, and 100 nm concentrations and analyzed 48 hours post-incubation. Percentage of F12 message remaining was determined by qPCR and were plotted against the control.

[0132] **Figure 3** is a graph depicting *in vitro* activity of F12 siRNA duplexes containing the modified phosphate prodrugs at the 5' end in primary mouse hepatocytes after transfection with RNAiMAX at 0.1, 1, and 10 nm concentrations and analyzed 24 hours post-transfection. Percentage of F12 message remaining was determined by qPCR and were plotted against the control.

[0133] **Figures 4A-J** show the representative LCMS spectra of oligonucleotides tested in the DTT reduction assay.

[0134] **Figure 5** is a graph depicting the relative mF12 protein in circulation by ELISA in mice following subcutaneous administration of F12 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at single dose 0.3 mg/kg, compared to PBS control.

[0135] **Figure 6** is a graph depicting the relative mF12 protein in circulation by ELISA in mice following subcutaneous administration of F12 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at single dose 0.1 mg/kg or 0.3 mg/kg, compared to PBS control.

[0136] **Figure 7** shows the possible *in vivo* cytosolic unmasking mechanism of the 5' cyclic disulfide modified phosphate prodrugs to reveal 5'-phosphate.

[0137] **Figure 8** is a graph depicting the relative SOD1 mRNA remaining in thoracic spinal cord, hippocampus, frontal cortex, striatum, and heart of rats, determined by qPCR, after 14 days following intrathecal (IT) administration of SOD1 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at a single dose of 0.1 mg.

[0138] **Figure 9** is a graph depicting the relative SOD1 mRNA remaining in thoracic spinal cord, cerebellum, frontal cortex, striatum, and heart of rats, determined by qPCR, after 84 days following intrathecal (IT) administration of SOD1 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at a single dose of 0.3 mg.

[0139] **Figure 10** is a graph depicting the relative SOD1 mRNA remaining in thoracic spinal cord, hippocampus, frontal cortex, striatum, and heart of rats, determined by qPCR, after 14 days following intrathecal (IT) administration of SOD1 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at a single dose of 0.9 mg.

[0140] **Figure 11** is a graph depicting the relative SOD1 mRNA remaining in thoracic spinal cord, cerebellum, frontal cortex, striatum, and heart of rats, determined by qPCR, after 84 days following intrathecal (IT) administration of SOD1 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at a single dose of 0.9 mg.

[0141] **Figure 12** is a graph depicting the relative SOD1 mRNA remaining by qPCR in thoracic spinal cord, hippocampus, frontal cortex, striatum, and heart of rats after 14 days following intrathecal administration of SOD1 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at a single dose of 0.9 mg.

[0142] **Figure 13** is a graph depicting the relative SOD1 mRNA remaining by qPCR in thoracic spinal cord, hippocampus, frontal cortex, striatum, and heart of rats after 14 days following intrathecal administration of SOD1 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at a single dose of either 0.3 mg or 0.9 mg.

[0143] **Figure 14** is a graph depicting the relative SOD1 mRNA remaining by qPCR in right brain hemisphere of mice after 7 days following intracranial ventricular administration of SOD1 siRNA duplexes containing the modified phosphate prodrugs at the 5' end at a single dose of 100 μg.

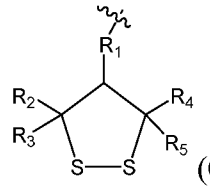
DETAILED DESCRIPTION

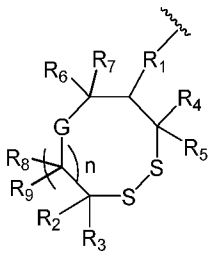
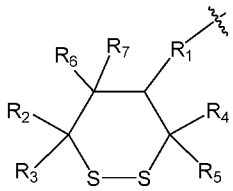
[0144] The inventors have discovered novel categories of cyclic disulfide moieties that can be introduced to the phosphate group of an oligonucleotide (e.g., a single-stranded iRNA agent a double-stranded iRNA agent) to temporarily mask the phosphate group, and that can

be *in vivo* cleaved via cellular activation. The cellular activation is via glutathione or dithiothreitol mediated reduction/bioconvention mechanism to release the active anionic form of the phosphate group from the masking group. The inventors have discovered that the cyclic disulfide moieties can be introduced at either the sense strand or the antisense strand or both the sense and antisense strands, at the 5' end, 3' end, and/or internal position(s) of a strand. Introduction of the cyclic disulfide moieties modified phosphate prodrug at the 5' end of the antisense strand provides particularly good results.

The modified phosphate prodrug compound

[0145] One aspect of the invention relates to a modified phosphate prodrug compound. The compound comprises a structure of formula (I): cyclic disulfide moiety — phosphorus coupling group (I).

[0146] The cyclic disulfide moiety has the structure of:  (C-I),

 (C-II), or  (C-III).

[0147] In formulas (C-I), (C-II), and (C-III):

R_1 is O or S, and is bonded to the P atom of the phosphorus coupling group;

R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

R_3 and R_5 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring;

G is O, N(R'), S, or $C(R^{14})(R^{15})$;

n is an integer of 0-6;

R^{13} is independently for each occurrence H, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, alkylcarbonyl, or arylcarbonyl, each of which can be optionally substituted with one or more R^{sub} groups;

R^{14}, R^{15}, and R^{16} are each independently H, halo, haloalkyl, alkyl, alkaryl, aryl, heteroaryl, aralkyl, hydroxy, alkyloxy, aryloxy, N(R')(R");

R' and R" are each independently H, alkyl, alkenyl, alkynyl, aryl, hydroxy, alkyloxy, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, or ω-hydroxy alkynyl, each of which can be optionally substituted with one or more R^{sub} groups; and

R^{sub} is independently for each occurrence halo, haloalkyl, alkyl, alkaryl, aryl, aralkyl, hydroxy, alkyloxy, aryloxy, oxo, nitro, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, alkylamino, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, arylcarbonyl, acyloxy, cyano, or ureido.

[0148] In some embodiments, in the cyclic disulfide moiety:

R_1 is O;

G is CH_2;

n is 0 or 1;

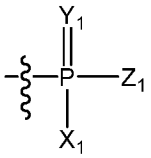
R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or C_1-C_6 alkylene-OR^{13}, N(R')(R") or C_1-C_6 alkylene-N(R')(R"), C_1-C_6 alkyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

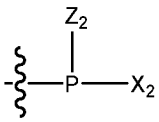
R_3 and R_5 are each independently H, halo, OR^{13} or C_1-C_6 alkylene-OR^{13}, N(R')(R") or C_1-C_6 alkylene-N(R')(R"), C_1-C_6 alkyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring of 6-8 atoms;

R^{13} is independently for each occurrence H, C_1-C_6 alkyl, aryl, alkylcarbonyl, or arylcarbonyl; and

R' and R" are each independently H or C_1-C_6 alkyl.

[0149] The phosphorus coupling group can have a structure of:



(P-I) or


(P-II).

[0150] In formulas (P-I) and (P-II):

X_1 and Z_1 are each independently H, OH, OM, OR^{13}, SH, SM, SR^{13}, C(O)H, S(O)H, or alkyl, each of which can be optionally substituted with one or more R^{sub} groups, N(R')(R"), $B(R^{13})_3$, BH_3^-, Se; or D-Q, wherein D is independently for each occurrence absent, O, S, N(R'), alkylene, each of which can be optionally substituted with one or more R^{sub} groups, and Q is independently for each occurrence a nucleoside or oligonucleotide;

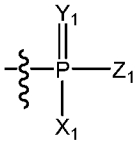
X_2 and Z_2 are each independently N(R')(R"), OR^{18}, or D-Q, wherein D is independently for each occurrence absent, O, S, N, N(R'), alkylene, each of which can be optionally substituted with one or more R^{sub} groups, and Q is independently for each occurrence a nucleoside or oligonucleotide,

Y_1 is S, O, or N(R');

M is an organic or inorganic cation; and

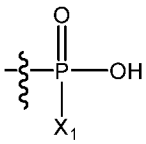
R^{18} is H or alkyl, optionally substituted with one or more R^{sub} groups.

[0151] In some embodiments, the phosphorus coupling group has the structure of



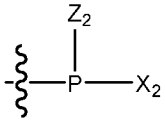
(P-I). In this formula: X_1 and Z_1 are each independently OH, OM, SH, SM, C(O)H, S(O)H, C_1-C_6 alkyl optionally substituted with one or more hydroxy or halo groups, or D-Q; D is independently for each occurrence absent, O, S, NH, C_1-C_6 alkylene optionally substituted with one or more halo groups; and Y_1 is S or O. In one embodiment, X_1 is OH or SH; and Z_1 is D-Q.

[0152] In one embodiment, the phosphorus coupling group has the structure of



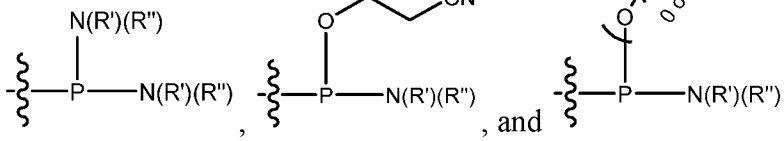
, wherein X_1 is OH or SH.

[0153] In some embodiments, the phosphorus coupling group has the structure of

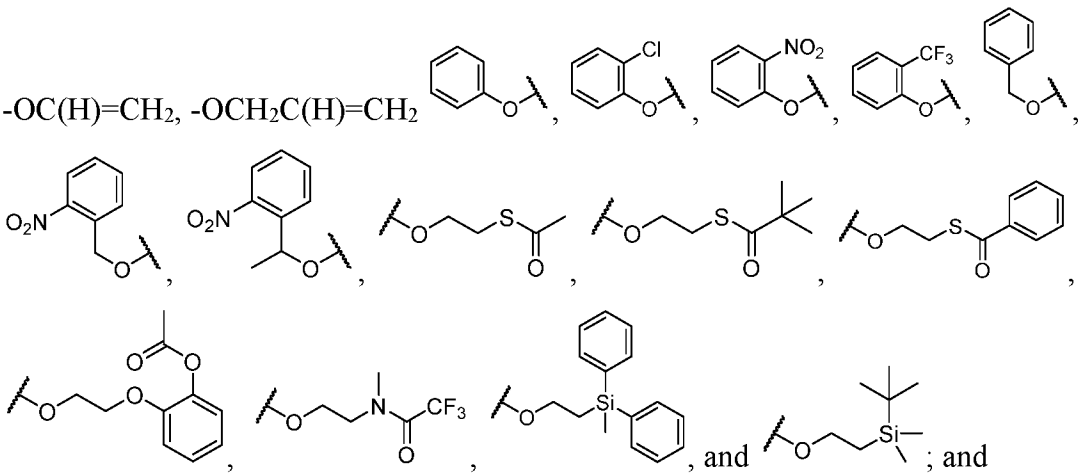


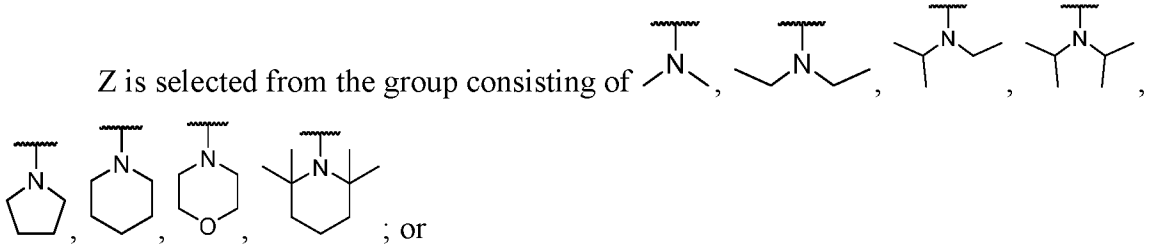
(P-II). In this formula, X_2 is N(R')(R"); Z_2 is X_2, OR^{18}, or D-Q; R^{18} is H or C_1-C_6 alkyl substituted with cyano; and R' and R'' are each independent C_1-C_6 alkyl (e.g., iso-propyl).

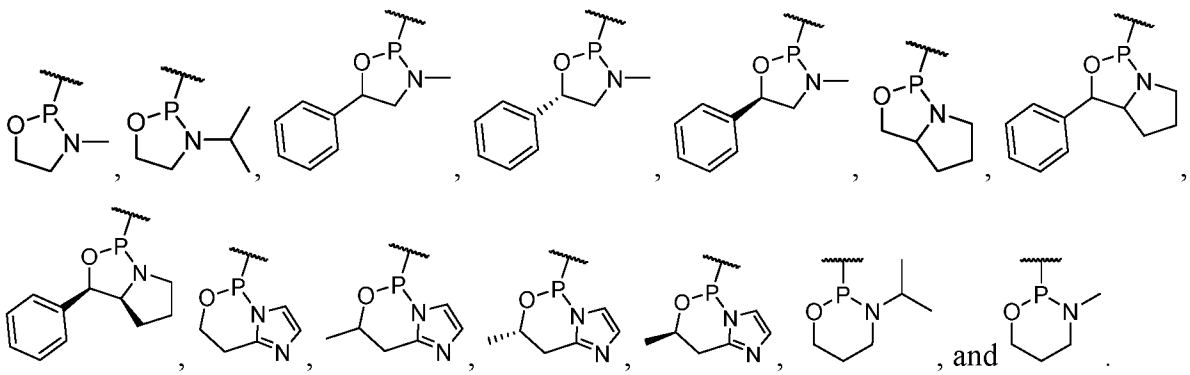
[0154] In one embodiment, the phosphorus coupling group has a structure selected from

the group consisting of  . The variables R', R'', and Q are defined as above in formulas P-I and P-II. In one embodiment, R' and R" are each iso-propyl.

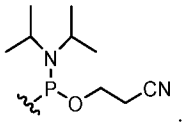
[0155] In some embodiments, the phosphorus coupling group has the structure -P(Z)(X), wherein:

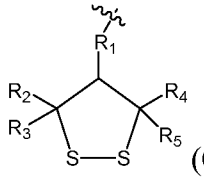
X is selected from the group consisting of $-OCH_3$, $-OCH_2CH_3$, $-OCH_2CH_2CH_3$, $-OCH_2CH(CH_3)_2$, $-OCH_2CH_2CN$, $-OCH_2CH_2Si(CH_3)_3$, $-OCH_2CH_2Si(CH_2CH_3)_3$, $-OC(H)=CH_2$, $-OCH_2C(H)=CH_2$  ; and

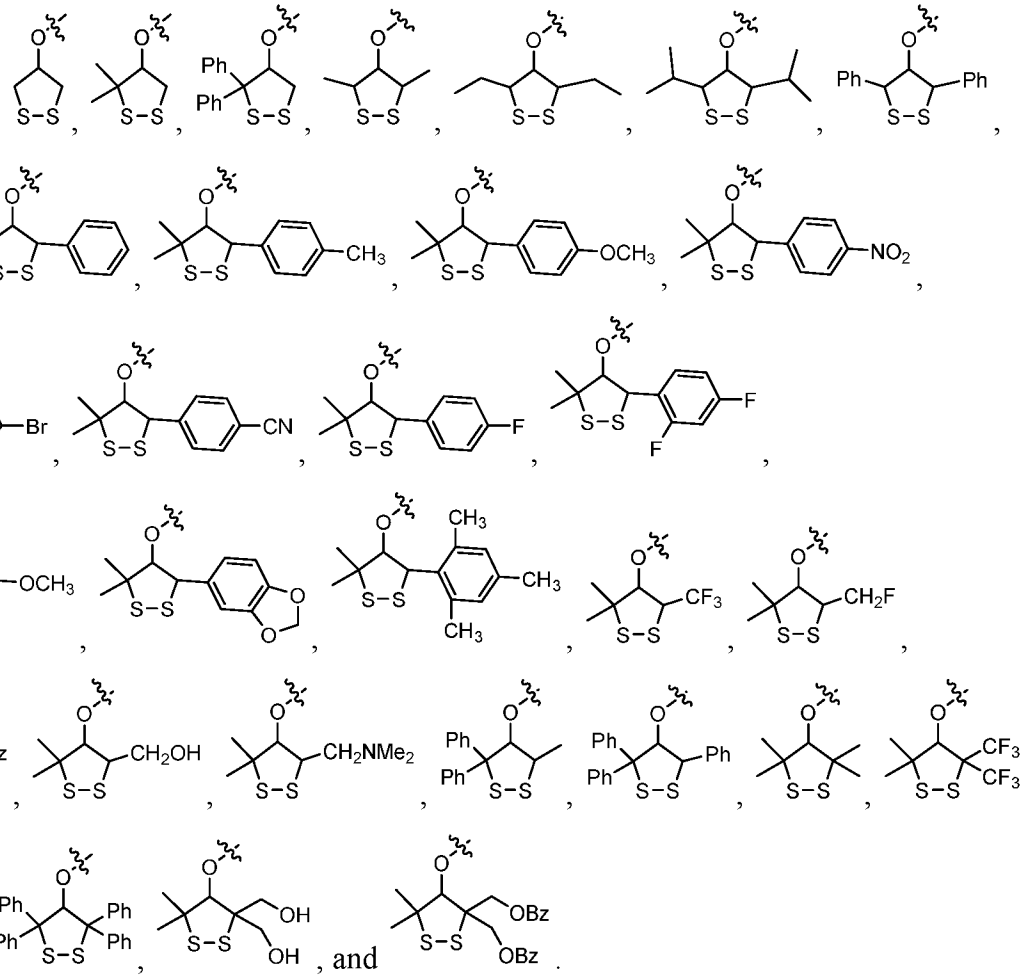
Z is selected from the group consisting of  ; or

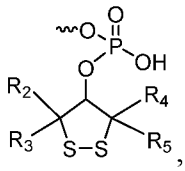
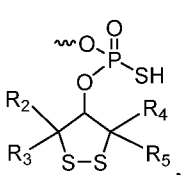
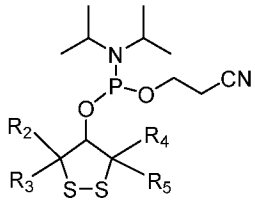
X and Z taken together with the phosphorus atom to which they are attached form a cyclic structure selected from the group consisting of  .

[0156] In one embodiment, the phosphorus coupling group has the structure of



[0157] The cyclic disulfide moiety can have the structure  (C-I).

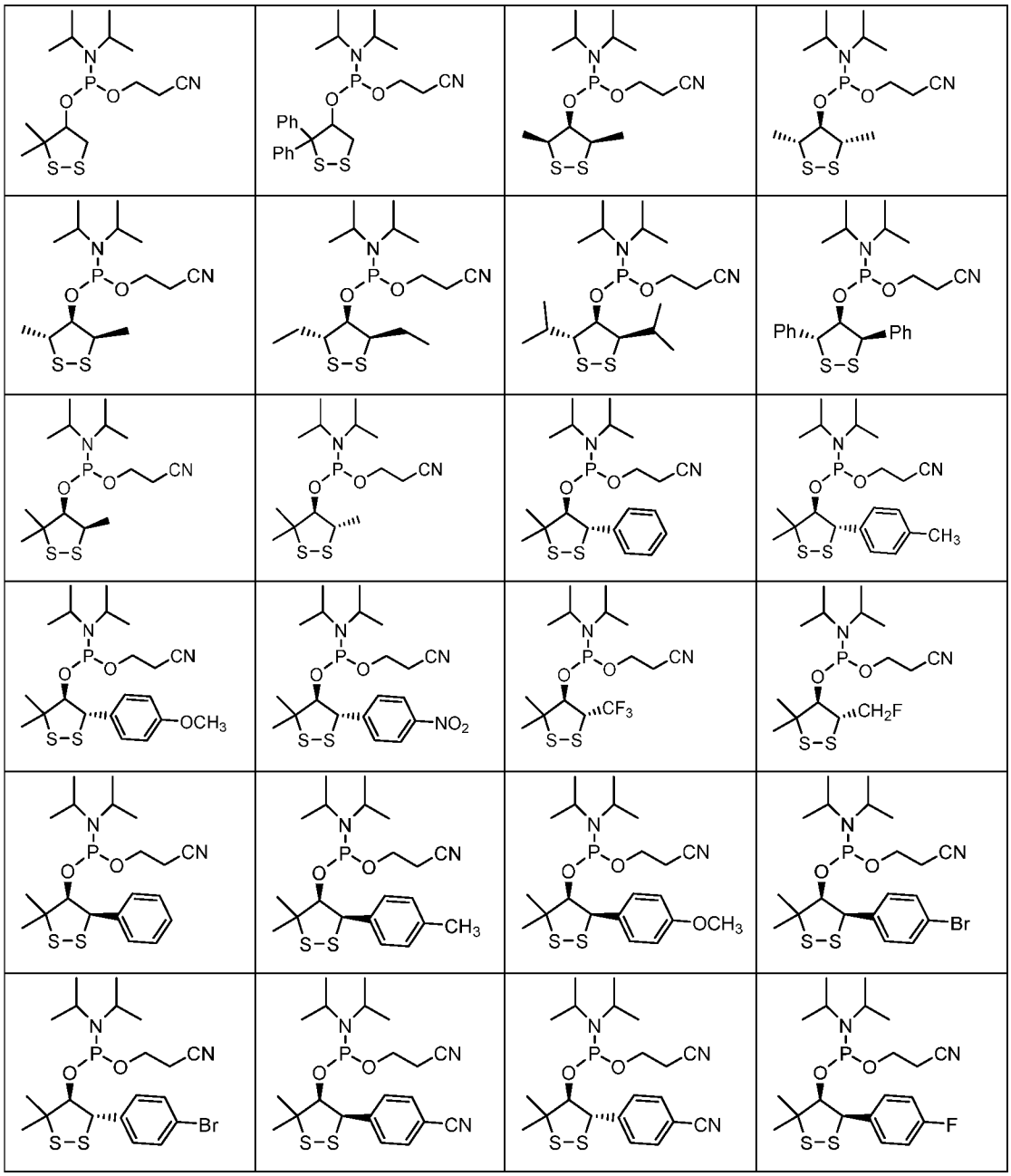
[0158] Exemplary cyclic disulfide moieties for 5-member cyclic compounds of formula (C-I) include:  , and .

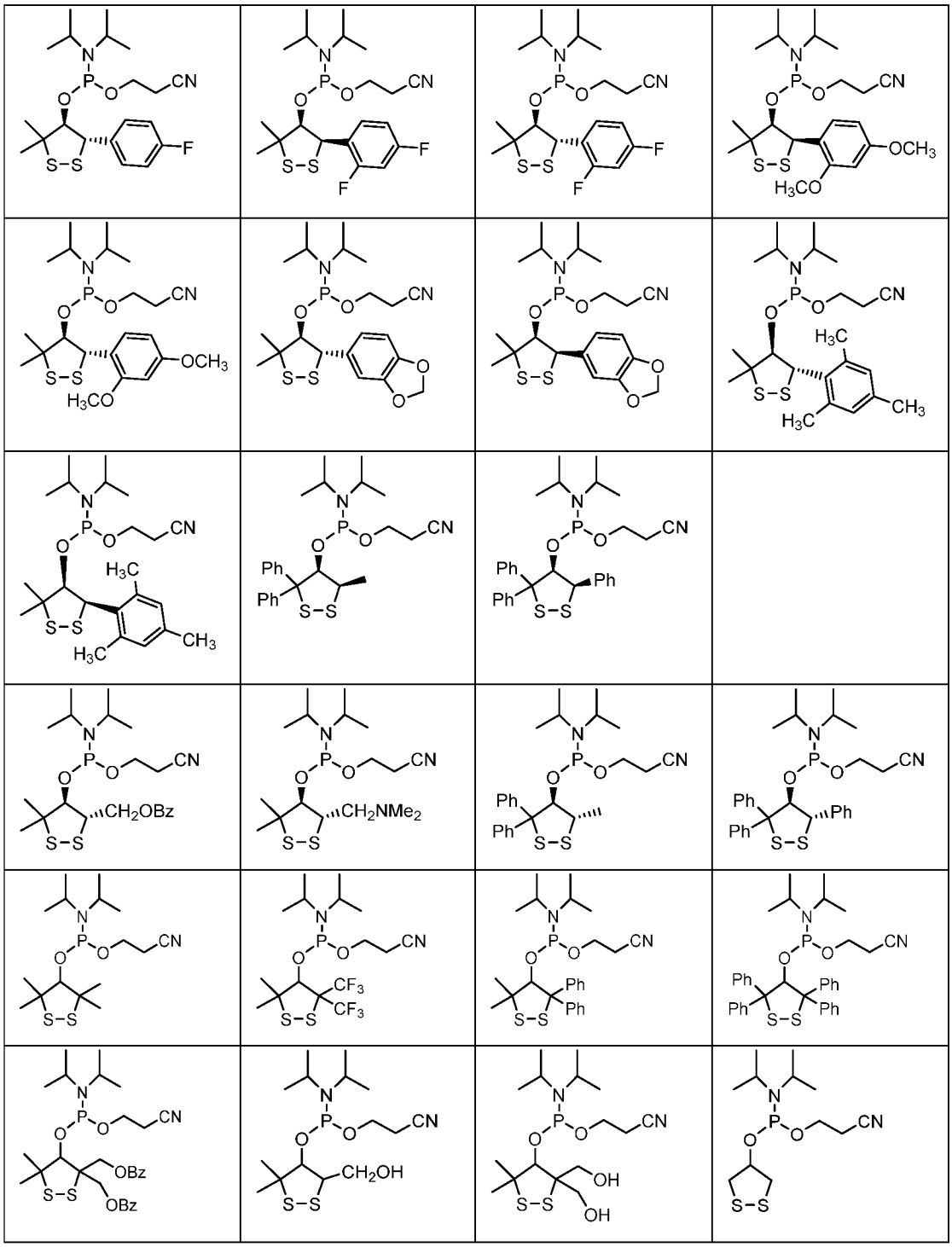
[0159] In some embodiments, the compound has the formula of  ,  , or  , wherein: R_2, R_3, R_4, and R_5 are each independently H,

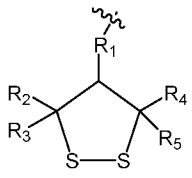
alkyl (e.g., CH_3), heterocyclic, CH_2R^{15}, aryl (e.g., phenyl), heteroaryl, $CHFR^{15}$, CF_2R^{15}, CF_3; and can be in any stereoisomeric configurations; and R^{15} is alkyl, heterocyclic, aryl, OH, O-alkyl, NH_2, NH(alkyl), $N(alkyl)_2$, CF_2 R^{15}, or CF_3; and can be in any stereoisomeric configurations.

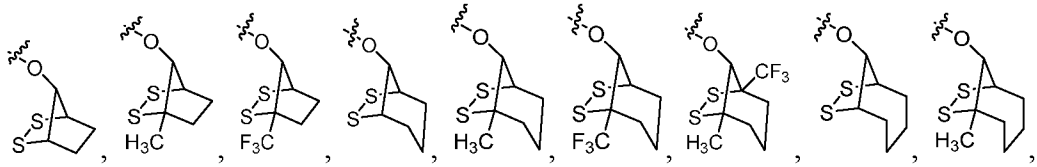
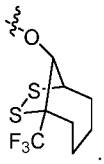
[0160] Exemplary compounds of formula (I) with a 5-member cyclic disulfide moiety are shown in Table 1.

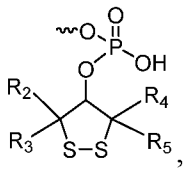
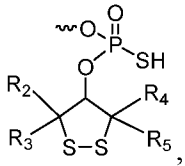
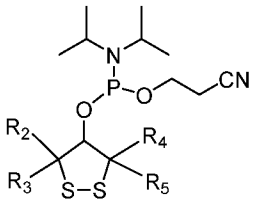
Table 1. Compounds with 5-member cyclic disulfide moiety





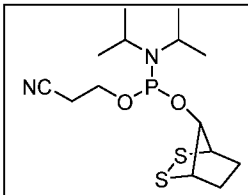
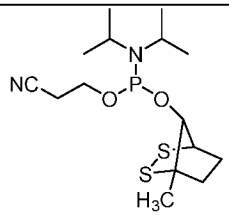
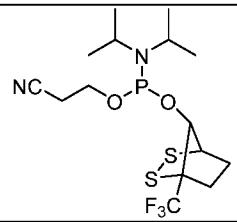
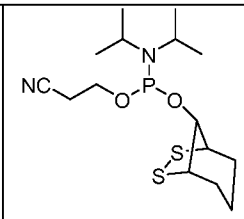
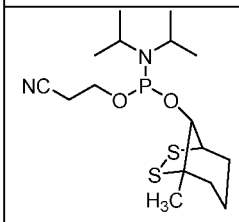
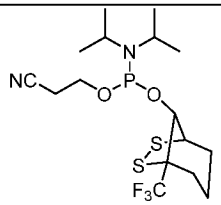
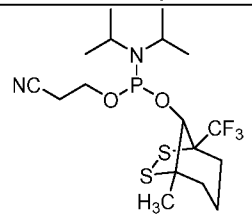
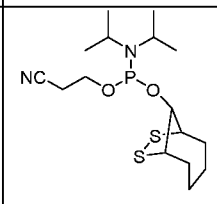
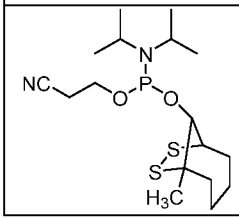
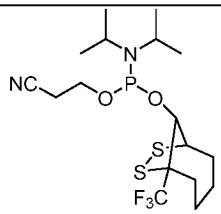
[0161] In some embodiments, the cyclic disulfide moiety has the structure  (C-I), wherein R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring. In one embodiment, the second ring has 6-8 atoms.

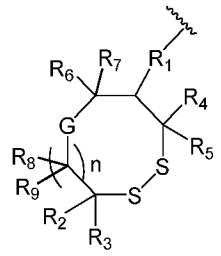
[0162] Exemplary cyclic disulfide moieties for bicyclic compounds of formula (C-I) include:  , and  .

[0163] In some embodiments, the compound has the formula of  ,  , or  , wherein R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring (e.g., having 6-8 atoms).

[0164] Exemplary compounds of formula (I) with a bicyclic disulfide moiety are shown in Table 2.

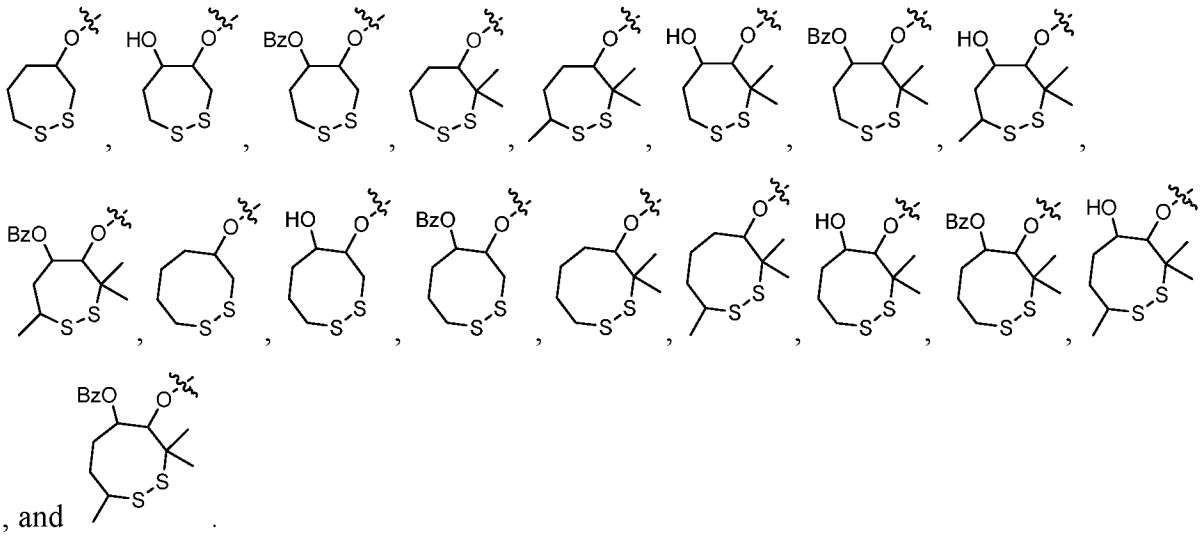
Table 2. Compounds with bicyclic disulfide moieties

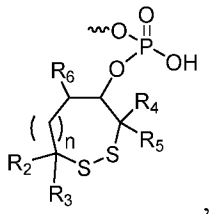


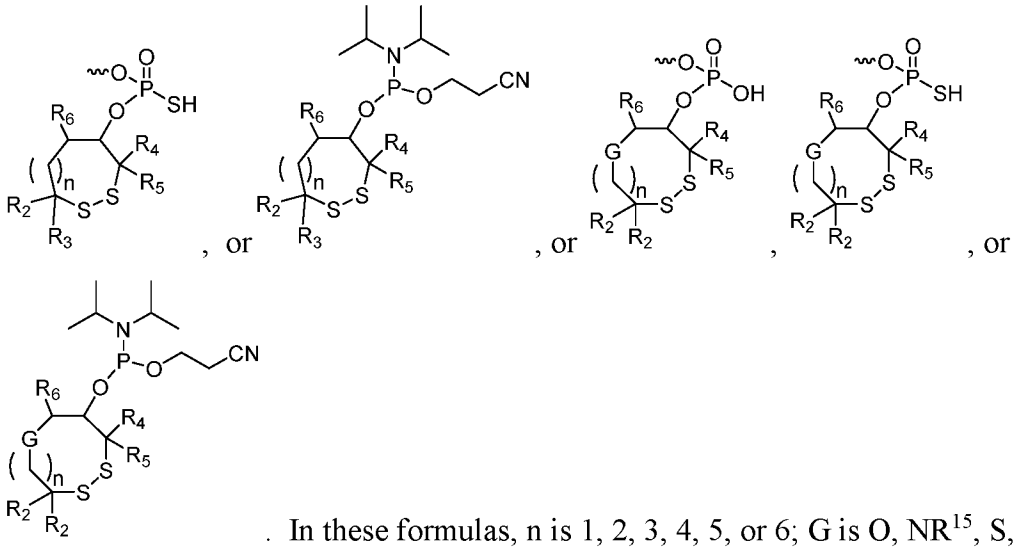
[0165] The cyclic disulfide moiety can also have the structure (C-II).

Exemplary cyclic disulfide moieties for a cyclic compounds of formula (C-II) include:



[0166] In some embodiments, the compound has the formula of:



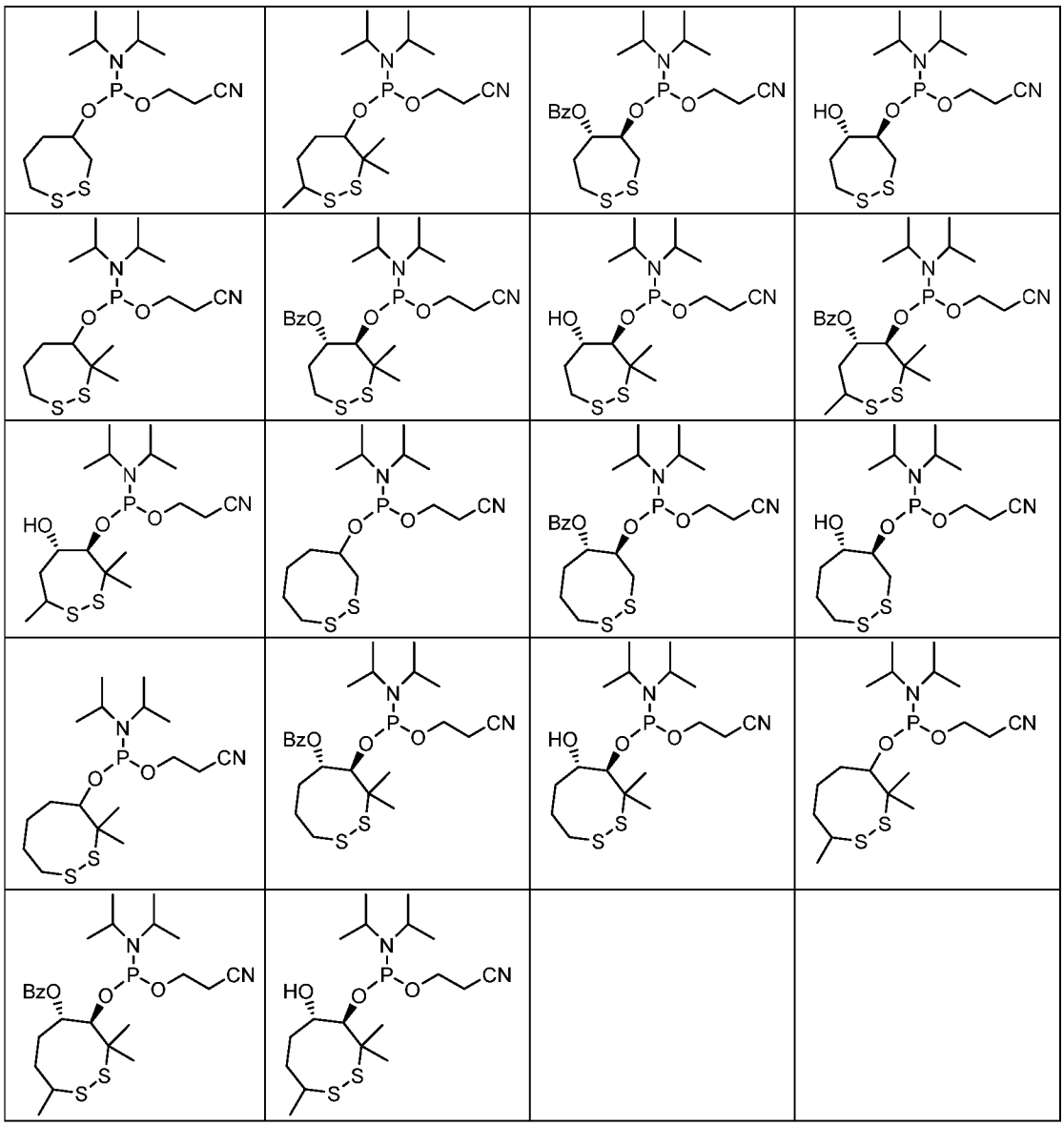


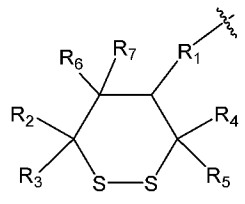
. In these formulas, n is 1, 2, 3, 4, 5, or 6; G is O, NR^{15}, S, or any other heteroatom; R_2, R_3, R_4, R_5, and R_6 are each independently H, alkyl (e.g., CH_3), heterocyclic, CH_2R^{15}, aryl (e.g., phenyl), heteroaryl, $CHFR^{15}$, CF_2R^{15}, CF_3; and can be in any stereoisomeric configurations; and R^{15} is alkyl, heterocyclic, aryl, OH, O-alkyl, NH_2,

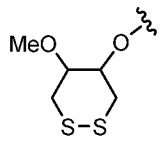
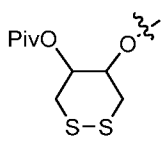
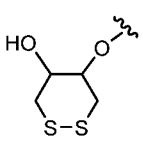
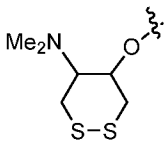
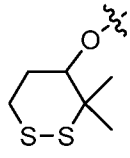
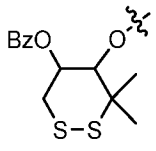
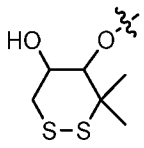
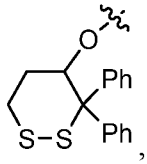
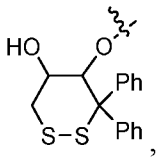
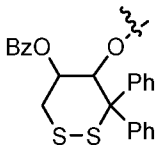
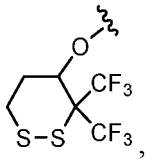
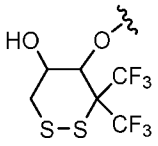
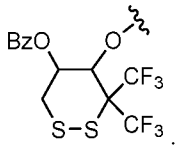
NH(alkyl), N(alkyl)$_2$, CF_2R^{15}, or CF_3; and can be in any stereoisomeric configurations.

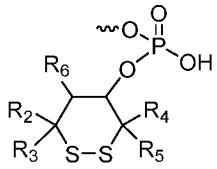
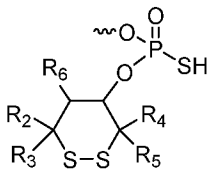
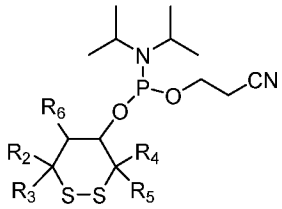
[0167] Exemplary compounds of formula (I) with a larger (7-member or larger) cyclic disulfide moiety are shown in Table 3.

Table 3. Compounds with 7- or 8- member cyclic disulfide moieties



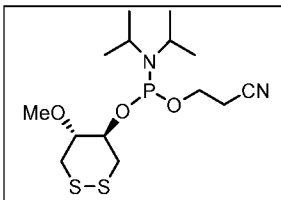
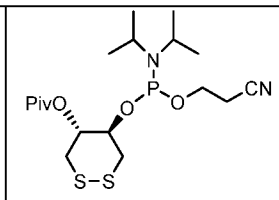
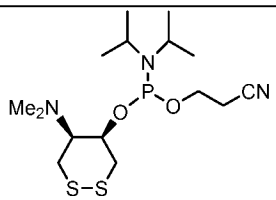
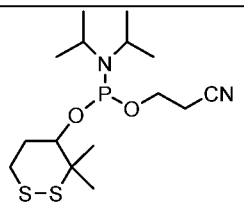
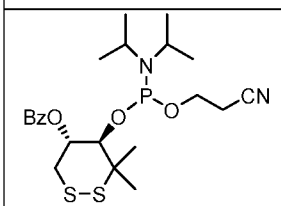
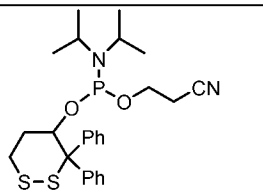
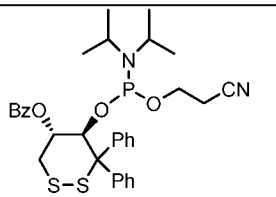
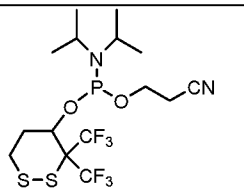
[0168] The cyclic disulfide moiety can also have the structure  (C-III). Exemplary cyclic disulfide moieties for a 6-member cyclic compounds of formula (C-III)

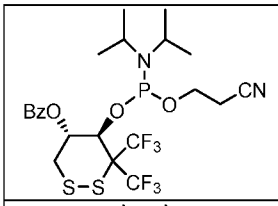
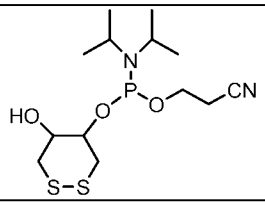
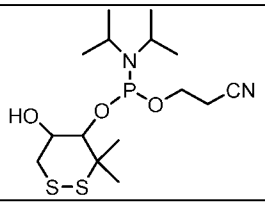
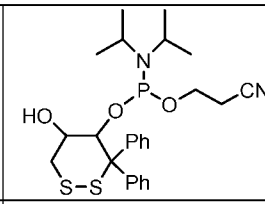
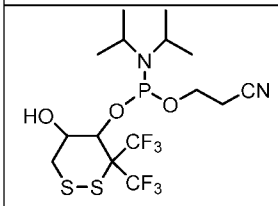
include:  ,  ,  ,  ,  ,  ,  ,  ,  ,  ,  ,  , and  .

[0169] In some embodiments, the compound has the formula of  ,  , or  . In this formula, R_2, R_3, R_4, R_5, and R_6 are each independently H, alkyl (e.g., CH_3), heterocyclic, CH_2R^{15}, aryl (e.g., phenyl), heteroaryl, $CHFR^{15}$, CF_2R^{15}, CF_3; and can be in any stereoisomeric configurations; and R^{15} is alkyl, heterocyclic, aryl, OH, O-alkyl, NH_2, NH(alkyl), N(alkyl)$_2$, CF_2 R^{15}, or CF_3; and can be in any stereoisomeric configurations.

[0170] Exemplary compounds of formula (I) with a 6-member cyclic disulfide moiety are shown in Table 4.

Table 4. Compounds with 7- or 8- member cyclic disulfide moieties

[0171] Certain terms are abbreviated within chemical structures throughout the application as would be familiar to those skilled in the art, including, e.g., methyl (Me), benzoyl (Bz), phenyl (Ph), and pivaloyl (Piv).

[0172] The term "halo" or "halogen" refers to any radical of fluorine, chlorine, bromine or iodine.

[0173] The term "aliphatic" or "aliphatic group," as used herein, means a straight-chain or branched, substituted or unsubstituted hydrocarbon chain that is saturated or contains one or more units of unsaturation, or a monocyclic hydrocarbon or bicyclic or polycyclic hydrocarbon that is saturated or contains one or more units of unsaturation, but is not aromatic, that has a single point of attachment to the rest of the molecule. In some embodiments, aliphatic groups contain 1-50 aliphatic carbon atoms, for instance, 1-10 aliphatic carbon atoms, 1-6 aliphatic carbon atoms, 1-5 aliphatic carbon atoms, 1-4 aliphatic carbon atoms, 1-3 aliphatic carbon atoms, or 1-2 aliphatic carbon atoms. In some embodiments, "cycloaliphatic" refers to a monocyclic or bicyclic C_3-C_{10} hydrocarbon (*e.g.*, a monocyclic C_3-C_6 hydrocarbon) that is saturated or contains one or more units of unsaturation, but is not aromatic, that has a single point of attachment to the rest of the molecule. Suitable aliphatic groups include, but are not limited to, linear or branched, substituted or unsubstituted alkyl, alkenyl, alkynyl groups and hybrids thereof such as (cycloalkyl)alkyl, (cycloalkenyl)alkyl, or (cycloalkyl)alkenyl.

[0174] The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C_1-C_{12} alkyl indicates that the group may have from 1 to 12 (inclusive) carbon atoms in it. Unless otherwise indicated, "alkyl" generally refers to C_1-C_{24} alkyl (e.g., C_1-C_{12} alkyl, C_1-C_8 alkyl, or C_1-C_4 alkyl). The term "haloalkyl" refers to an alkyl in which one or more hydrogen atoms are replaced by halo, and includes alkyl moieties in which all hydrogens have been

replaced by halo (e.g., perfluoroalkyl). Alkyl and haloalkyl groups may be optionally inserted with O, N, or S. The terms “aralkyl” refers to an alkyl moiety in which an alkyl hydrogen atom is replaced by an aryl group. Aralkyl includes groups in which more than one hydrogen atom has been replaced by an aryl group. Examples of “aralkyl” include benzyl, 9-fluorenyl, benzhydryl, and trityl groups.

[0175] The term "alkenyl" refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more double bonds. Unless otherwise indicated, “alkenyl” generally refers to C_2-C_8 alkenyl (e.g., C_2-C_6 alkenyl, C_2-C_4 alkenyl, or C_2-C_3 alkenyl). Examples of a typical alkenyl include, but not limited to, allyl, propenyl, 2-butenyl, 3-hexenyl and 3-octenyl groups. The term "alkynyl" refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more triple bonds. Unless otherwise indicated, “alkynyl” generally refers to C_2-C_8 alkynyl (e.g., C_2-C_6 alkynyl, C_2-C_4 alkynyl, or C_2-C_3 alkynyl). Some examples of a typical alkynyl are ethynyl, 2-propynyl, and 3-methylbutynyl, and propargyl. The sp^2 and sp^3 carbons may optionally serve as the point of attachment of the alkenyl and alkynyl groups, respectively.

[0176] The term “alkoxy” refers to an -O-alkyl radical. The term “alkylene” refers to a divalent alkyl (*i.e.*, -R-). The term “aminoalkyl” refers to an alkyl substituted with an amino. The term “mercapto” refers to an -SH radical. The term “thioalkoxy” refers to an -S-alkyl radical.

[0177] The term “alkylene” refers to a bivalent alkyl group. An “alkylene chain” is a polymethylene group, i.e., —$(CH_2)_n$—, wherein n is a positive integer, preferably from 1 to 6, from 1 to 4, from 1 to 3, from 1 to 2, or from 2 to 3. A substituted alkylene chain is a polymethylene group in which one or more methylene hydrogen atoms are replaced with a substituent. Suitable substituents include those described below.

[0178] The term “alkenylene” refers to a bivalent alkenyl group. A substituted alkenylene chain is a polymethylene group containing at least one double bond in which one or more hydrogen atoms are replaced with a substituent. Suitable substituents include those described below.

[0179] The term “aryl” refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic ring system wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. The term “aryl” may be used interchangeably with the term “aryl ring.” Examples of aryl groups include phenyl, biphenyl, naphthyl, anthracyl, and the like, which may bear one or more substituents. Also included within the scope of the term “aryl,” as it is used herein, is a

group in which an aromatic ring is fused to one or more non-aromatic rings, such as indanyl, phthalimidyl, naphthimidyl, phenanthridinyl, or tetrahydronaphthyl, and the like. The term "arylalkyl" or the term "aralkyl" refers to alkyl substituted with an aryl. The term "arylalkoxy" refers to an alkoxy substituted with aryl.

[0180] The term "cycloalkyl" or "cyclyl" as employed herein includes saturated and partially unsaturated, but not aromatic, cyclic hydrocarbon groups having 3 to 12 carbons, for example, 3 to 8 carbons, and, for example, 3 to 6 carbons, wherein the cycloalkyl group additionally may be optionally substituted. Cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

[0181] The term "heteroaryl" or "heteroar-" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. The term also include groups in which a heteroaromatic ring is fused to one or more aryl, cycloalkyl, or heterocyclyl rings, where the radical or point of attachment is on the heteroaromatic ring. Examples of heteroaryl groups include pyrrolyl, pyridyl, pyridazinyl, pyrazolyl, triazolyl, tetrazolyl, oxazolyl, isoxazolyl, oxadiazolyl, furanyl, imidazolyl, benzimidazolyl, pyrimidinyl, pyrazinyl, indolizinyl, thiophenyl or thienyl, quinolinyl, indolyl, thiazolyl, isothiazolyl, thiadiazolyl, purinyl, naphthyridinyl, pteridinyl, isoindolyl, benzothienyl, benzofuranyl, dibenzofuranyl, indazolyl, benzthiazolyl, quinolyl, isoquinolyl, cinnolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, 4H-quinolizinyl, carbazolyl, acridinyl, phenazinyl, phenothiazinyl, phenoxazinyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, and pyrido[2,3-b]-1,4-oxazin-3(4H)-one and the like. The term "heteroarylalkyl" or the term "heteroaralkyl" refers to an alkyl substituted with a heteroaryl. The term "heteroarylalkoxy" refers to an alkoxy substituted with heteroaryl.

[0182] The term "heterocyclyl," "heterocycle," "heterocyclic radical," or "heterocyclic ring" refers to a nonaromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or

tricyclic, respectively), wherein 0, 1, 2 or 3 atoms of each ring may be substituted by a substituent. When used in reference to a ring atom of a heterocycle, the term “nitrogen” includes a substituted nitrogen. As an example, in a saturated or partially unsaturated ring having 0-3 heteroatoms selected from oxygen, sulfur or nitrogen, the nitrogen may be N (as in 3,4-dihydro-2H-pyrrolyl), NH (as in pyrrolidinyl), or +NR (as in N-substituted pyrrolidinyl). Examples of heterocyclyl groups include trizolyl, tetrazolyl, piperazinyl, pyrrolidinyl, dioxanyl, dioxolanyl, diazepinyl, oxazepinyl, thiazepinyl, morpholinyl, tetrahydrofuranyl, tetrahydrothiophenyl pyrrolidinyl, piperidinyl, pyrrolinyl, tetrahydroquinolinyl, tetrahydroisoquinolinyl, decahydroquinolinyl, oxazolidinyl, quinuclidinyl, and the like. The term “heterocyclylalkyl” refers to an alkyl group substituted by a heterocyclyl, wherein the alkyl and heterocyclyl portions independently are optionally substituted.

[0183] The term “oxo” refers to an oxygen atom, which forms a carbonyl when attached to carbon, an N-oxide when attached to nitrogen, and a sulfoxide or sulfone when attached to sulfur.

[0184] The term “acyl” refers to an alkylcarbonyl, cycloalkylcarbonyl, arylcarbonyl, heterocyclylcarbonyl, or heteroarylcarbonyl substituent, any of which may be further substituted by substituents.

[0185] The term "substituted" refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent including, but not limited to: halo, alkyl, alkenyl, alkynyl, aryl, heterocyclyl, thiol, alkylthio, arylthio, alkylthioalkyl, arylthioalkyl, alkylsulfonyl, alkylsulfonylalkyl, arylsulfonylalkyl, alkoxy, aryloxy, aralkoxy, aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, alkoxycarbonyl, aryloxycarbonyl, haloalkyl, amino, trifluoromethyl, cyano, nitro, alkylamino, arylamino, alkylaminoalkyl, arylaminoalkyl, aminoalkylamino, hydroxy, alkoxyalkyl, carboxyalkyl, alkoxycarbonylalkyl, aminocarbonylalkyl, acyl, aralkoxycarbonyl, carboxylic acid, sulfonic acid, sulfonyl, phosphonic acid, aryl, heteroaryl, heterocyclic, and aliphatic. It is understood that the substituent can be further substituted.

[0186] Suitable divalent substituents on a saturated carbon atom of an “optionally substituted” group include the following: =O, =S, =NNR*$_2$, =NNHC(O)R*, =NNHC(O)OR*, =NNHS(O)$_2$R*, =NR*, =NOR*, —O(C(R*$_2$))$_{2\text{-}3}$O—, or —S(C(R*$_2$))$_{2\text{-}3}$S—, wherein each independent occurrence of R* is selected from hydrogen, $C_{1\text{-}6}$ aliphatic which may be substituted as defined below, or an unsubstituted 5-6-membered saturated,

partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur. Suitable divalent substituents that are bound to vicinal substitutable carbons of an "optionally substituted" group include: —$O(CR^*_2)_{2-3}O$—, wherein each independent occurrence of R* is selected from hydrogen, C_{1-6} aliphatic which may be substituted as defined below, or an unsubstituted 5-6-membered saturated, partially unsaturated, or aryl ring having 0-4 heteroatoms independently selected from nitrogen, oxygen, or sulfur.

The oligonucleotide prodrug

[0187] Another aspect of the invention relates to an oligonucleotide (e.g., a single-stranded iRNA agent or a double-stranded iRNA agent) comprising one or more compounds that comprise the structure of formula (I): cyclic disulfide moiety — phosphorus coupling group (I). In formula (I), at least one phosphorus coupling group contains a nucleoside or oligonucleotide.

[0188] All the above embodiments relating to all the formulas of the cyclic disulfide moiety, all the formulas of the phosphorus coupling group, all the variables defined in these formulas, and all the subgenus and species structures relating to the compound, the cyclic disulfide moiety, and the phosphorus coupling group in the first aspect of the invention relating to the compound (or modified phosphate prodrug compound) are suitable in this aspect of the invention relating to the oligonucleotide.

[0189] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end of the oligonucleotide.

[0190] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at the 3'-end of the oligonucleotide.

[0191] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at an internal position of the oligonucleotide.

[0192] In some embodiments, when the cyclic disulfide moiety has the structure of formula (C-III), at least one cyclic disulfide moiety is connected at the 5' end of the nucleoside or oligonucleotide.

[0193] Additional structures for the modified phosphate prodrug compound include those disclosed in WO 2014/088920, published on June 12, 2014, the content of which is incorporated herein by reference in its entirety. In particular, these modified phosphate

prodrug compounds are incorporated into the oligonucleotide at the 5' end.

[0194] In some embodiments, the oligonucleotide is a single-stranded oligonucleotide, such as a single-stranded iRNA agent (e.g., single-stranded siRNA).

[0195] In some embodiments, the oligonucleotide is a double-stranded oligonucleotide, such as a double-stranded iRNA agent (e.g., double-stranded siRNA), comprising a sense strand and an antisense strand.

[0196] In one embodiment, the sense strand contains at least one cyclic disulfide moiety. In one embodiment, the antisense strand contains at least one cyclic disulfide moiety. In one embodiment, both the sense strand and the antisense strand each contain at least one cyclic disulfide moiety.

[0197] Introduction of the cyclic disulfide moiety to the phosphate group as a temporary protecting group, on either the sense or antisense strand or both the sense and antisense strands, are illustrated in Schemes 10-15 in Example 9 below.

Oligonucleotide Definitions and Designs

[0198] Unless specific definitions are provided, the nomenclature utilized in connection with, and the procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques may be used for chemical synthesis, and chemical analysis. Certain such techniques and procedures may be found for example in "Carbohydrate Modifications in Antisense Research" Edited by Sangvi and Cook, American Chemical Society, Washington D.C., 1994; "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., 18th edition, 1990; and "Antisense Drug Technology, Principles, Strategies, and Applications" Edited by Stanley T. Crooke, CRC Press, Boca Raton, Fla.; and Sambrook et al., "Molecular Cloning, A laboratory Manual," 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, which are hereby incorporated by reference for any purpose. Where permitted, all patents, applications, published applications and other publications and other data referred to throughout in the disclosure herein are incorporated by reference in their entirety.

[0199] As used herein, the term "target nucleic acid" refers to any nucleic acid molecule the expression or activity of which is capable of being modulated by an siRNA compound. Target nucleic acids include, but are not limited to, RNA (including, but not limited to pre-mRNA and mRNA or portions thereof) transcribed from DNA encoding a target protein, and

also cDNA derived from such RNA, and miRNA. For example, the target nucleic acid can be a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state. In some embodiments, a target nucleic acid can be a nucleic acid molecule from an infectious agent.

[0200] As used herein, the term "iRNA" refers to an agent that mediates the targeted cleavage of an RNA transcript. These agents associate with a cytoplasmic multi-protein complex known as RNAi-induced silencing complex (RISC). Agents that are effective in inducing RNA interference are also referred to as siRNA, RNAi agent, or iRNA agent, herein. Thus, these terms can be used interchangeably herein. As used herein, the term iRNA includes microRNAs and pre-microRNAs. Moreover, the "compound" or "compounds" of the invention as used herein, also refers to the iRNA agent, and can be used interchangeably with the iRNA agent.

[0201] The iRNA agent should include a region of sufficient homology to the target gene, and be of sufficient length in terms of nucleotides, such that the iRNA agent, or a fragment thereof, can mediate downregulation of the target gene. (For ease of exposition the term nucleotide or ribonucleotide is sometimes used herein in reference to one or more monomeric subunits of an iRNA agent. It will be understood herein that the usage of the term "ribonucleotide" or "nucleotide", herein can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions.) Thus, the iRNA agent is or includes a region which is at least partially, and in some embodiments fully, complementary to the target RNA. It is not necessary that there be perfect complementarity between the iRNA agent and the target, but the correspondence must be sufficient to enable the iRNA agent, or a cleavage product thereof, to direct sequence specific silencing, *e.g.*, by RNAi cleavage of the target RNA, *e.g.*, mRNA. Complementarity, or degree of homology with the target strand, is most critical in the antisense strand. While perfect complementarity, particularly in the antisense strand, is often desired some embodiments can include, particularly in the antisense strand, one or more, or for example, 6, 5, 4, 3, 2, or fewer mismatches (with respect to the target RNA). The sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double stranded character of the molecule.

[0202] iRNA agents include: molecules that are long enough to trigger the interferon response (which can be cleaved by Dicer (Bernstein *et al.* 2001. Nature, 409:363-366) and enter a RISC (RNAi-induced silencing complex)); and, molecules which are sufficiently short

that they do not trigger the interferon response (which molecules can also be cleaved by Dicer and/or enter a RISC), *e.g.*, molecules which are of a size which allows entry into a RISC, *e.g.*, molecules which resemble Dicer-cleavage products. Molecules that are short enough that they do not trigger an interferon response are termed siRNA agents or shorter iRNA agents herein. "siRNA agent or shorter iRNA agent" as used herein, refers to an iRNA agent, *e.g.*, a double stranded RNA agent or single strand agent, that is sufficiently short that it does not induce a deleterious interferon response in a human cell, *e.g.*, it has a duplexed region of less than 60, 50, 40, or 30 nucleotide pairs. The siRNA agent, or a cleavage product thereof, can down regulate a target gene, *e.g.*, by inducing RNAi with respect to a target RNA, wherein the target may comprise an endogenous or pathogen target RNA.

[0203] A "single strand iRNA agent" as used herein, is an iRNA agent which is made up of a single molecule. It may include a duplexed region, formed by intra-strand pairing, *e.g.*, it may be, or include, a hairpin or pan-handle structure. Single strand iRNA agents may be antisense with regard to the target molecule. A single strand iRNA agent may be sufficiently long that it can enter the RISC and participate in RISC mediated cleavage of a target mRNA. A single strand iRNA agent is at least 14, and in other embodiments at least 15, 20, 25, 29, 35, 40, or 50 nucleotides in length. In certain embodiments, it is less than 200, 100, or 60 nucleotides in length.

[0204] A loop refers to a region of an iRNA strand that is unpaired with the opposing nucleotide in the duplex when a section of the iRNA strand forms base pairs with another strand or with another section of the same strand.

[0205] Hairpin iRNA agents will have a duplex region equal to or at least 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region will may be equal to or less than 200, 100, or 50, in length. In certain embodiments, ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. The hairpin may have a single strand overhang or terminal unpaired region, in some embodiments at the 3', and in certain embodiments on the antisense side of the hairpin. In some embodiments, the overhangs are 2-3 nucleotides in length.

[0206] A "double stranded (ds) iRNA agent" as used herein, is an iRNA agent which includes more than one, and in some cases two, strands in which interchain hybridization can form a region of duplex structure.

[0207] As used herein, the terms "siRNA activity" and "RNAi activity" refer to gene

silencing by an siRNA.

[0208] As used herein, "gene silencing" by a RNA interference molecule refers to a decrease in the mRNA level in a cell for a target gene by at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99% up to and including 100%, and any integer in between of the mRNA level found in the cell without the presence of the miRNA or RNA interference molecule. In one preferred embodiment, the mRNA levels are decreased by at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 99%, up to and including 100% and any integer in between 5% and 100%."

[0209] As used herein the term "modulate gene expression" means that expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

[0210] As used herein, gene expression modulation happens when the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits is at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2-fold, 3-fold, 4-fold, 5-fold or more different from that observed in the absence of the siRNA, e.g., RNAi agent. The % and/or fold difference can be calculated relative to the control or the non-control, for example,

$$\%\ \text{difference} = \frac{[\text{expression with siRNA} - \text{expression without siRNA}]}{\text{expression without siRNA}}$$

or

$$\%\ \text{difference} = \frac{[\text{expression with siRNA} - \text{expression without siRNA}]}{\text{expression without siRNA}}$$

[0211] As used herein, the term "inhibit", "down-regulate", or "reduce" in relation to gene expression, means that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of

modulator. The gene expression is down-regulated when expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced at least 10% lower relative to a corresponding non-modulated control, and preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99% or most preferably, 100% (i.e., no gene expression).

[0212] As used herein, the term "increase" or "up-regulate" in relation to gene expression means that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is increased above that observed in the absence of modulator. The gene expression is up-regulated when expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is increased at least 10% relative to a corresponding non-modulated control, and preferably at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 100%, 1.1-fold, 1.25-fold, 1.5-fold, 1.75-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 50-fold, 100-fold or more.

[0213] The term "increased" or "increase" as used herein generally means an increase by a statically significant amount; for the avoidance of any doubt, "increased" means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

[0214] The term "reduced" or "reduce" as used herein generally means a decrease by a statistically significant amount. However, for avoidance of doubt, "reduced" means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (i.e. absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level.

[0215] The double-stranded iRNAs comprise two oligonucleotide strands that are

sufficiently complementary to hybridize to form a duplex structure. Generally, the duplex structure is between 15 and 30, more generally between 18 and 25, yet more generally between 19 and 24, and most generally between 19 and 21 base pairs in length. In some embodiments, longer double-stranded iRNAs of between 25 and 30 base pairs in length are preferred. In some embodiments, shorter double-stranded iRNAs of between 10 and 15 base pairs in length are preferred. In another embodiment, the double-stranded iRNA is at least 21 nucleotides long.

[0216] In some embodiments, the double-stranded iRNA comprises a sense strand and an antisense strand, wherein the antisense RNA strand has a region of complementarity which is complementary to at least a part of a target sequence, and the duplex region is 14-30 nucleotides in length. Similarly, the region of complementarity to the target sequence is between 14 and 30, more generally between 18 and 25, yet more generally between 19 and 24, and most generally between 19 and 21 nucleotides in length.

[0217] The phrase “antisense strand” as used herein, refers to an oligonucleotide strand that is substantially or 100% complementary to a target sequence of interest. The phrase "antisense strand" includes the antisense region of both oligonucleotide strands that are formed from two separate strands, as well as unimolecular oligonucleotide strands that are capable of forming hairpin or dumbbell type structures. The terms “antisense strand” and “guide strand” are used interchangeably herein.

[0218] The phrase “sense strand” refers to an oligonucleotide strand that has the same nucleoside sequence, in whole or in part, as a target sequence such as a messenger RNA or a sequence of DNA. The terms “sense strand” and “passenger strand” are used interchangeably herein.

[0219] By “specifically hybridizable” and "complementary" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non- traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al, 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci.* USA 83:9373-9377; Turner et al., 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing)

with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9,10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" or 100% complementarity means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. Less than perfect complementarity refers to the situation in which some, but not all, nucleoside units of two strands can hydrogen bond with each other. "Substantial complementarity" refers to polynucleotide strands exhibiting 90% or greater complementarity, excluding regions of the polynucleotide strands, such as overhangs, that are selected so as to be noncomplementary. Specific binding requires 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 performed. The non-target sequences typically differ by at least 5 nucleotides.

[0220] In some embodiments, the double-stranded region is equal to or at least, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotide pairs in length.

[0221] In some embodiments, the antisense strand is equal to or at least 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[0222] In some embodiments, the sense strand is equal to or at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[0223] In one embodiment, the sense and antisense strands are each 15 to 30 nucleotides in length. In one embodiment, the sense and antisense strands are each 19 to 25 nucleotides in length. In one embodiment, the sense and antisense strands are each 21 to 23 nucleotides in length.

[0224] In some embodiments, one strand has at least one stretch of 1-5 single-stranded nucleotides in the double-stranded region. By "stretch of single-stranded nucleotides in the double-stranded region" is meant that there is present at least one nucleotide base pair at both ends of the single-stranded stretch. In some embodiments, both strands have at least one stretch of 1-5 (e.g., 1, 2, 3, 4, or 5) single-stranded nucleotides in the double stranded region. When both strands have a stretch of 1-5 (e.g., 1, 2, 3, 4, or 5) single-stranded nucleotides in the double stranded region, such single-stranded nucleotides can be opposite to each other (e.g., a stretch of mismatches) or they can be located such that the second strand has no single-stranded nucleotides opposite to the single-stranded iRNAs of the first strand and vice

versa (e.g., a single-stranded loop). In some embodiments, the single-stranded nucleotides are present within 8 nucleotides from either end, for example 8, 7, 6, 5, 4, 3, or 2 nucleotide from either the 5' or 3' end of the region of complementarity between the two strands.

[0225] In one embodiment, the oligonucleotide comprises a single-stranded overhang on at least one of the termini. In one embodiment, the single-stranded overhang is 1, 2, or 3 nucleotides in length.

[0226] In one embodiment, the sense strand of the iRNA agent is 21- nucleotides in length, and the antisense strand is 23-nucleotides in length, wherein the strands form a double-stranded region of 21 consecutive base pairs having a 2-nucleotide long single-stranded overhangs at the 3'-end.

[0227] In some embodiments, each strand of the double-stranded iRNA has a ZXY structure, such as is described in PCT Publication No. 2004080406, which is hereby incorporated by reference in its entirety.

[0228] In certain embodiment, the two strands of double-stranded oligonucleotide can be linked together. The two strands can be linked to each other at both ends, or at one end only. By linking at one end is meant that 5'-end of first strand is linked to the 3'-end of the second strand or 3'-end of first strand is linked to 5'-end of the second strand. When the two strands are linked to each other at both ends, 5'-end of first strand is linked to 3'-end of second strand and 3'-end of first strand is linked to 5'-end of second strand. The two strands can be linked together by an oligonucleotide linker including, but not limited to, $(N)_n$; wherein N is independently a modified or unmodified nucleotide and n is 3-23. In some embodiments, n is 3-10, e.g., 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the oligonucleotide linker is selected from the group consisting of GNRA, $(G)_4$, $(U)_4$, and $(dT)_4$, wherein N is a modified or unmodified nucleotide and R is a modified or unmodified purine nucleotide. Some of the nucleotides in the linker can be involved in base-pair interactions with other nucleotides in the linker. The two strands can also be linked together by a non-nucleosidic linker, e.g. a linker described herein. It will be appreciated by one of skill in the art that any oligonucleotide chemical modifications or variations describe herein can be used in the oligonucleotide linker.

[0229] Hairpin and dumbbell type oligonucleotide will have a duplex region equal to or at least 14, 15, 15, 16, 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region can be equal to or less than 200, 100, or 50, in length. In some embodiments, ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. .

[0230] The hairpin oligonucleotide can have a single strand overhang or terminal unpaired region, in some embodiments at the 3', and in some embodiments on the antisense side of the hairpin. In some embodiments, the overhangs are 1-4, more generally 2-3 nucleotides in length. The hairpin oligonucleotide s that can induce RNA interference are also referred to as "shRNA" herein.

[0231] In certain embodiments, two oligonucleotide strands specifically hybridize when there is a sufficient degree of complementarity to avoid non-specific binding of the antisense strand to non-target nucleic acid sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and under conditions in which assays are performed in the case of in vitro assays.

[0232] As used herein, "stringent hybridization conditions" or "stringent conditions" refers to conditions under which an antisense strand will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances, and "stringent conditions" under which antisense strand hybridize to a target sequence are determined by the nature and composition of the antisense strand and the assays in which they are being investigated.

[0233] It is understood in the art that incorporation of nucleotide affinity modifications may allow for a greater number of mismatches compared to an unmodified oligonucleotide. Similarly, certain oligonucleotide sequences may be more tolerant to mismatches than other oligonucleotide sequences. One of ordinary skill in the art is capable of determining an appropriate number of mismatches between oligonucleotides, or between an oligonucleotide and a target nucleic acid, such as by determining melting temperature (Tm). Tm or ΔTm can be calculated by techniques that are familiar to one of ordinary skill in the art. For example, techniques described in Freier et al. (Nucleic Acids Research, 1997, 25, 22: 4429-4443) allow one of ordinary skill in the art to evaluate nucleotide modifications for their ability to increase the melting temperature of an RNA:DNA duplex.

Additional dsRNA Design

[0234] In one embodiment, the iRNA agent is a double ended bluntmer of 19 nt in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 7, 8, 9 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

[0235] In one embodiment, the iRNA agent is a double ended bluntmer of 20 nt in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 8, 9, 10 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

[0236] In one embodiment, the iRNA agent is a double ended bluntmer of 21 nt in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

[0237] In one embodiment, the iRNA agent comprises a 21 nucleotides (nt) sense strand and a 23 nucleotides (nt) antisense, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'end; the antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end, wherein one end of the iRNA is blunt, while the other end is comprises a 2 nt overhang. Preferably, the 2 nt overhang is at the 3'-end of the antisense. Optionally, the iRNA agent further comprises a ligand (e.g., $GalNAc_3$).

[0238] In one embodiment, the iRNA agent comprises a sense and antisense strands, wherein: the sense strand is 25-30 nucleotide residues in length, wherein starting from the 5' terminal nucleotide (position 1) positions 1 to 23 of said first strand comprise at least 8 ribonucleotides; antisense strand is 36-66 nucleotide residues in length and, starting from the 3' terminal nucleotide, comprises at least 8 ribonucleotides in the positions paired with positions 1- 23 of sense strand to form a duplex; wherein at least the 3 ' terminal nucleotide of antisense strand is unpaired with sense strand, and up to 6 consecutive 3' terminal nucleotides are unpaired with sense strand, thereby forming a 3' single stranded overhang of 1-6 nucleotides; wherein the 5' terminus of antisense strand comprises from 10-30 consecutive nucleotides which are unpaired with sense strand, thereby forming a 10-30 nucleotide single stranded 5' overhang; wherein at least the sense strand 5' terminal and 3' terminal nucleotides are base paired with nucleotides of antisense strand when sense and antisense strands are aligned for maximum complementarity, thereby forming a substantially duplexed region between sense and antisense strands; and antisense strand is sufficiently complementary to a target RNA along at least 19 ribonucleotides of antisense strand length to reduce target gene

expression when said double stranded nucleic acid is introduced into a mammalian cell; and wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at or near the cleavage site.

[0239] In one embodiment, the iRNA agent comprises a sense and antisense strands, wherein said iRNA agent comprises a first strand having a length which is at least 25 and at most 29 nucleotides and a second strand having a length which is at most 30 nucleotides with at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at position 11, 12, 13 from the 5' end; wherein said 3' end of said first strand and said 5' end of said second strand form a blunt end and said second strand is 1-4 nucleotides longer at its 3' end than the first strand, wherein the duplex region which is at least 25 nucleotides in length, and said second strand is sufficiently complementary to a target mRNA along at least 19 nt of said second strand length to reduce target gene expression when said iRNA agent is introduced into a mammalian cell, and wherein dicer cleavage of said iRNA preferentially results in an siRNA comprising said 3' end of said second strand, thereby reducing expression of the target gene in the mammal. Optionally, the iRNA agent further comprises a ligand (e.g., $GalNAc_3$).

[0240] In one embodiment, the sense strand contains at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at the cleavage site in the sense strand. For instance, the sense strand can contain at least one motif of three 2'-F modifications on three consecutive nucleotides within 7-15 positions from the 5'end.

[0241] In one embodiment, the antisense strand can also contain at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at or near the cleavage site in the antisense strand. For instance, the antisense strand can contain at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides within 9-15 positions from the 5'end.

[0242] For iRNA agent having a duplex region of 17-23 nt in length, the cleavage site of the antisense strand is typically around the 10, 11 and 12 positions from the 5'-end. Thus the motifs of three identical modifications may occur at the 9, 10, 11 positions; 10, 11, 12 positions; 11, 12, 13 positions; 12, 13, 14 positions; or 13, 14, 15 positions of the antisense strand, the count starting from the 1^{st} nucleotide from the 5'-end of the antisense strand, or,

the count starting from the 1st paired nucleotide within the duplex region from the 5'- end of the antisense strand. The cleavage site in the antisense strand may also change according to the length of the duplex region of the iRNA from the 5'-end.

[0243] In some embodiments, the iRNA agent comprises a sense strand and antisense strand each having 14 to 30 nucleotides, wherein the sense strand contains at least two motifs of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site within the strand and at least one of the motifs occurs at another portion of the strand that is separated from the motif at the cleavage site by at least one nucleotide. In one embodiment, the antisense strand also contains at least one motif of three identical modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site within the strand. The modification in the motif occurring at or near the cleavage site in the sense strand is different than the modification in the motif occurring at or near the cleavage site in the antisense strand.

[0244] In some embodiments, the iRNA agent comprises a sense strand and antisense strand each having 14 to 30 nucleotides, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site in the strand. In one embodiment, the antisense strand also contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at or near the cleavage site.

[0245] In some embodiments, the iRNA agent comprises a sense strand and antisense strand each having 14 to 30 nucleotides, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'end, and wherein the antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

[0246] In one embodiment, the iRNA agent comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mismatch can occur in the overhang region or the duplex region. The base pair can be ranked on the basis of their propensity to promote dissociation or melting (e.g., on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation: A:U is preferred over G:C; G:U is preferred over G:C; and I:C is preferred over G:C (I=inosine). Mismatches, e.g., non-canonical or other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings; and pairings which include a

universal base are preferred over canonical pairings.

[0247] In one embodiment, the iRNA agent comprises at least one of the first 1, 2, 3, 4, or 5 base pairs within the duplex regions from the 5'- end of the antisense strand can be chosen independently from the group of: A:U, G:U, I:C, and mismatched pairs, e.g., non-canonical or other than canonical pairings or pairings which include a universal base, to promote the dissociation of the antisense strand at the 5'-end of the duplex.

[0248] In one embodiment, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from the group consisting of A, dA, dU, U, and dT. Alternatively, at least one of the first 1, 2 or 3 base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair. For example, the first base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair.

[0249] In one embodiment, 100%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35% or 30% of the dsRNA agent is modified. For example, when 50% of the dsRNA agent is modified, 50% of all nucleotides present in the dsRNA agent contain a modification as described herein.

[0250] In some embodiments, the oligonucleotide contains one or more 2'-O modifications selected from the group consisting of 2'-deoxy, 2'-O-methoxyalkyl, 2'-O-methyl, 2'-O-allyl, 2'-C-allyl, 2'-fluoro, 2'-O-N-methylacetamido (2'-O-NMA), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), 2'-O-aminopropyl (2'-O-AP), and 2'-ara-F.

[0251] In one embodiment, each of the sense and antisense strands is independently modified with non-natural nucleotides such as acyclic nucleotides, LNA, HNA, CeNA, 2'-methoxyethyl, 2'- O-methyl, 2'-O-allyl, 2'-C-allyl, 2'-deoxy, 2'-fluoro, 2'-O-N-methylacetamido (2'-O-NMA), a 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), 2'-O-aminopropyl (2'-O-AP), or 2'-ara-F.

[0252] In one embodiment, each of the sense and antisense strands of the dsRNA agent contains at least two different modifications.

[0253] In some embodiments, the oligonucleotide contains one, two, three, four, five, six, seven, eight, nine, ten, eleven or twelve 2'-F modification(s). In one example, oligonucleotide contains nine or ten 2'-F modifications.

[0254] In one embodiment, the oligonucleotide does not contain any 2'-F modification.

[0255] The iRNA agent may further comprise at least one phosphorothioate or methylphosphonate internucleotide linkage. The phosphorothioate or methylphosphonate internucleotide linkage modification may occur on any nucleotide of the sense strand or

antisense strand or both in any position of the strand. For instance, the internucleotide linkage modification may occur on every nucleotide on the sense strand or antisense strand; each internucleotide linkage modification may occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand may contain both internucleotide linkage modifications in an alternating pattern. The alternating pattern of the internucleotide linkage modification on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the internucleotide linkage modification on the sense strand may have a shift relative to the alternating pattern of the internucleotide linkage modification on the antisense strand.

[0256] In one embodiment, the iRNA comprises the phosphorothioate or methylphosphonate internucleotide linkage modification in the overhang region. For example, the overhang region may contain two nucleotides having a phosphorothioate or methylphosphonate internucleotide linkage between the two nucleotides. Internucleotide linkage modifications also may be made to link the overhang nucleotides with the terminal paired nucleotides within duplex region. For example, at least 2, 3, 4, or all the overhang nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage, and optionally, there may be additional phosphorothioate or methylphosphonate internucleotide linkages linking the overhang nucleotide with a paired nucleotide that is next to the overhang nucleotide. For instance, there may be at least two phosphorothioate internucleotide linkages between the terminal three nucleotides, in which two of the three nucleotides are overhang nucleotides, and the third is a paired nucleotide next to the overhang nucleotide. Preferably, these terminal three nucleotides may be at the 3'-end of the antisense strand.

[0257] In one embodiment, the sense strand and/or antisense strand comprises one or more blocks of phosphorothioate or methylphosphonate internucleotide linkages. In one example, the sense strand comprises one block of two phosphorothioate or methylphosphonate internucleotide linkages. In one example, the antisense strand comprises two blocks of two phosphorothioate or methylphosphonate internucleotide linkages. For example, the two blocks of phosphorothioate or methylphosphonate internucleotide linkages are separated by 16-18 phosphate internucleotide linkages.

[0258] In one embodiment, each of the sense and antisense strands has 15-30 nucleotides. In one example, the sense strand has 19-22 nucleotides, and the antisense strand has 19-25 nucleotides. In another example, the sense strand has 21 nucleotides, and the antisense strand

has 23 nucleotides.

[0259] In one embodiment, the nucleotide at position 1 of the 5'-end of the antisense strand in the duplex is selected from the group consisting of A, dA, dU, U, and dT. In one embodiment, at least one of the first, second, and third base pair from the 5'-end of the antisense strand is an AU base pair.

[0260] In one embodiment, the antisense strand of the dsRNA agent is 100% complementary to a target RNA to hybridize thereto and inhibits its expression through RNA interference. In another embodiment, the antisense strand of the dsRNA agent is at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, or at least 50% complementary to a target RNA.

[0261] In one aspect, the invention relates to a dsRNA agent as defined herein capable of inhibiting the expression of a target gene. The dsRNA agent comprises a sense strand and an antisense strand, each strand having 14 to 40 nucleotides. The sense strand contains at least one thermally destabilizing nucleotide, wherein at least one of said thermally destabilizing nucleotide occurs at or near the site that is opposite to the seed region of the antisense strand (i.e. at position 2-8 of the 5'-end of the antisense strand).

[0262] The thermally destabilizing nucleotide can occur, for example, between positions 14-17 of the 5'-end of the sense strand when the sense strand is 21 nucleotides in length. The antisense strand contains at least two modified nucleic acids that are smaller than a sterically demanding 2'-OMe modification. Preferably, the two modified nucleic acids that are smaller than a sterically demanding 2'-OMe are separated by 11 nucleotides in length. For example, the two modified nucleic acids are at positions 2 and 14 of the 5'end of the antisense strand.

[0263] In one embodiment, the dsRNA agents comprise:

(a) a sense strand having:
  (i) a length of 18-23 nucleotides;
  (ii) three consecutive 2'-F modifications at positions 7-15; and

(b) an antisense strand having:
  (i) a length of 18-23 nucleotides;
  (ii) at least 2'-F modifications anywhere on the strand; and
  (iii) at least two phosphorothioate internucleotide linkages at the first five nucleotides (counting from the 5' end);

wherein the dsRNA agents have one or more lipophilic monomers containing one or more lipophilic moieties conjugated to one or more positions on at least one strand; and either have

two nucleotides overhang at the 3'-end of the antisense strand, and a blunt end at the 5'-end of the antisense strand; or blunt end both ends of the duplex.

[0264] In one embodiment, the dsRNA agents comprise:

(a) a sense strand having:
  (i) a length of 18-23 nucleotides;
  (ii) less than four 2'-F modifications;

(b) an antisense strand having:
  (i) a length of 18-23 nucleotides;
  (ii) at less than twelve 2'-F modification; and
  (iii) at least two phosphorothioate internucleotide linkages at the first five nucleotides (counting from the 5' end);

wherein the dsRNA agents have one or more lipophilic monomers containing one or more lipophilic moieties conjugated to one or more positions on at least one strand; and either have two nucleotides overhang at the 3'-end of the antisense strand, and a blunt end at the 5'-end of the antisense strand; or blunt end both ends of the duplex.

[0265] In one embodiment, the dsRNA agents comprise:

(a) a sense strand having:
  (i) a length of 19-35 nucleotides;
  (ii) less than four 2'-F modifications;

(b) an antisense strand having:
  (i) a length of 19-35 nucleotides;
  (ii) at less than twelve 2'-F modification; and
  (iii) at least two phosphorothioate internucleotide linkages at the first five nucleotides (counting from the 5' end);

wherein the duplex region is between 19 to 25 base pairs (preferably 19, 20, 21 or 22); and wherein the dsRNA agents have one or more lipophilic monomers containing one or more lipophilic moieties conjugated to one or more positions on at least one strand; and either have two nucleotides overhang at the 3'-end of the antisense strand, and a blunt end at the 5'-end of the antisense strand; or blunt end both ends of the duplex.

[0266] In one embodiment, the dsRNA agents comprise a sense strand and antisense strands having a length of 15-30 nucleotides; at least two phosphorothioate internucleotide linkages at the first five nucleotides on the antisense strand (counting from the 5' end); wherein the duplex region is between 19 to 25 base pairs (preferably 19, 20, 21 or 22);

wherein the dsRNA agents have one or more lipophilic monomers containing one or more lipophilic moieties conjugated to one or more positions on at least one strand; and wherein the dsRNA agents have less than 20% , less than 15% and less than 10% non-natural nucleotide.

[0267] In one embodiment, the dsRNA agents comprise a sense strand and antisense strands having a length of 15-30 nucleotides; at least two phosphorothioate internucleotide linkages at the first five nucleotides on the antisense strand (counting from the 5' end); wherein the duplex region is between 19 to 25 base pairs (preferably 19, 20, 21 or 22); wherein the dsRNA agents have one or more lipophilic monomers containing one or more lipophilic moieties conjugated to one or more positions on at least one strand; and wherein the dsRNA agents have greater than 80% , greater than 85% and greater than 90% natural nucleotide, such as 2'-OH, 2'-deoxy and 2'-OMe are natural nucleotides.

[0268] In one embodiment, the dsRNA agents comprise a sense strand and antisense strands having a length of 15-30 nucleotides; at least two phosphorothioate internucleotide linkages at the first five nucleotides on the antisense strand (counting from the 5' end); wherein the duplex region is between 19 to 25 base pairs (preferably 19, 20, 21 or 22); wherein the dsRNA agents have one or more lipophilic monomers containing one or more lipophilic moieties conjugated to one or more positions on at least one strand; and wherein the dsRNA agents have 100% natural nucleotide, such as 2'-OH, 2'-deoxy and 2'-OMe are natural nucleotides.

[0269] In one embodiment, the dsRNA agents comprises a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, wherein the sense strand sequence is represented by formula (I):

$$5'\ n_p\text{-}N_a\text{-}(X\ X\ X)_i\text{-}N_b\text{-}Y\ Y\ Y\ \text{-}N_b\text{-}(Z\ Z\ Z)_j\text{-}N_a\text{-}n_q\ 3' \quad (I)$$

wherein:

i and j are each independently 0 or 1;

p and q are each independently 0-6;

each N_a independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b independently represents an oligonucleotide sequence comprising 1, 2, 3, 4, 5, or 6 modified nucleotides;

each n_p and n_q independently represent an overhang nucleotide;

wherein N_b and Y do not have the same modification;

wherein XXX, YYY and ZZZ each independently represent one motif of three identical modifications on three consecutive nucleotides;

wherein the dsRNA agents have one or more lipophilic monomers containing one or more lipophilic moieties conjugated to one or more positions on at least one strand; and

wherein the antisense strand of the dsRNA comprises two blocks of one, two or three phosphorothioate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 phosphate internucleotide linkages.

[0270] Various publications described multimeric siRNA and can all be used with the iRNA of the invention. Such publications include WO2007/091269, US Patent No. 7858769, WO2010/141511, WO2007/117686, WO2009/014887 and WO2011/031520, which are hereby incorporated by reference in their entirety.

[0271] In some embodiments, the antisense strand is 100% complementary to a target RNA to hybridize thereto and inhibits its expression through RNA interference. In another embodiment, the antisense strand is at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, or at least 50% complementary to a target RNA.

Nucleic acid modifications

[0272] In some embodiments, the oligonucleotide comprises at least one nucleic acid modification described herein. For example, at least one modification selected from the group consisting of modified internucleoside linkage, modified nucleobase, modified sugar, and any combinations thereof. Without limitations, such a modification can be present anywhere in the oligonucleotide. For example, the modification can be present in one of the RNA molecules.

Nucleic acid modifications (Nucleobases)

[0273] The naturally occurring base portion of a nucleoside is typically a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. For those nucleosides that include a pentofuranosyl sugar, a phosphate group can be linked to the 2′, 3′ or 5′ hydroxyl moiety of the sugar. In forming oligonucleotides, those phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. Within oligonucleotides, the phosphate groups are commonly referred

to as forming the internucleoside backbone of the oligonucleotide. The naturally occurring linkage or backbone of RNA and of DNA is a 3′ to 5′ phosphodiester linkage.

[0274] In addition to "unmodified" or "natural" nucleobases such as the purine nucleobases adenine (A) and guanine (G), and the pyrimidine nucleobases thymine (T), cytosine (C) and uracil (U), many modified nucleobases or nucleobase mimetics known to those skilled in the art are amenable with the oligonucleotides described herein. The unmodified or natural nucleobases can be modified or replaced to provide iRNAs having improved properties. For example, nuclease resistant oligonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the oligomer modifications described herein. Alternatively, substituted or modified analogs of any of the above bases and "universal bases" can be employed. When a natural base is replaced by a non-natural and/or universal base, the nucleotide is said to comprise a modified nucleobase and/or a nucleobase modification herein. Modified nucleobase and/or nucleobase modifications also include natural, non-natural and universal bases, which comprise conjugated moieties, e.g. a ligand described herein. Preferred conjugate moieties for conjugation with nucleobases include cationic amino groups which can be conjugated to the nucleobase via an appropriate alkyl, alkenyl or a linker with an amide linkage.

[0275] An oligonucleotide described herein can also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Exemplary modified nucleobases include, but are not limited to, other synthetic and natural nucleobases such as inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, 2-(halo)adenine, 2-(alkyl)adenine, 2-(propyl)adenine, 2-(amino)adenine, 2-(aminoalkyl)adenine, 2-(aminopropyl)adenine, 2-(methylthio)-N^6-(isopentenyl)adenine, 6-(alkyl)adenine, 6-(methyl)adenine, 7-(deaza)adenine, 8-(alkenyl)adenine, 8-(alkyl)adenine, 8-(alkynyl)adenine, 8-(amino)adenine, 8-(halo)adenine, 8-(hydroxyl)adenine, 8-(thioalkyl)adenine, 8-(thiol)adenine, N^6-(isopentyl)adenine, N^6-(methyl)adenine, N^6, N^6-(dimethyl)adenine, 2-(alkyl)guanine,2-(propyl)guanine, 6-(alkyl)guanine, 6-(methyl)guanine, 7-(alkyl)guanine, 7-(methyl)guanine, 7-(deaza)guanine, 8-(alkyl)guanine, 8-(alkenyl)guanine, 8-(alkynyl)guanine, 8-(amino)guanine, 8-(halo)guanine, 8-(hydroxyl)guanine, 8-(thioalkyl)guanine, 8-(thiol)guanine, N-(methyl)guanine, 2-

(thio)cytosine, 3-(deaza)-5-(aza)cytosine, 3-(alkyl)cytosine, 3-(methyl)cytosine, 5-(alkyl)cytosine, 5-(alkynyl)cytosine, 5-(halo)cytosine, 5-(methyl)cytosine, 5-(propynyl)cytosine, 5-(propynyl)cytosine, 5-(trifluoromethyl)cytosine, 6-(azo)cytosine, N^4-(acetyl)cytosine, 3-(3-amino-3-carboxypropyl)uracil, 2-(thio)uracil, 5-(methyl)-2-(thio)uracil, 5-(methylaminomethyl)-2-(thio)uracil, 4-(thio)uracil, 5-(methyl)-4-(thio)uracil, 5-(methylaminomethyl)-4-(thio)uracil, 5-(methyl)-2,4-(dithio)uracil, 5-(methylaminomethyl)-2,4-(dithio)uracil, 5-(2-aminopropyl)uracil, 5-(alkyl)uracil, 5-(alkynyl)uracil, 5-(allylamino)uracil, 5-(aminoallyl)uracil, 5-(aminoalkyl)uracil, 5-(guanidiniumalkyl)uracil, 5-(1,3-diazole-1-alkyl)uracil, 5-(cyanoalkyl)uracil, 5-(dialkylaminoalkyl)uracil, 5-(dimethylaminoalkyl)uracil, 5-(halo)uracil, 5-(methoxy)uracil, uracil-5-oxyacetic acid, 5-(methoxycarbonylmethyl)-2-(thio)uracil, 5-(methoxycarbonyl-methyl)uracil, 5-(propynyl)uracil, 5-(propynyl)uracil, 5-(trifluoromethyl)uracil, 6-(azo)uracil, dihydrouracil, N^3-(methyl)uracil, 5-uracil (*i.e.*, pseudouracil), 2-(thio)pseudouracil,4-(thio)pseudouracil,2,4-(dithio)psuedouracil,5-(alkyl)pseudouracil, 5-(methyl)pseudouracil, 5-(alkyl)-2-(thio)pseudouracil, 5-(methyl)-2-(thio)pseudouracil, 5-(alkyl)-4-(thio)pseudouracil, 5-(methyl)-4-(thio)pseudouracil, 5-(alkyl)-2,4-(dithio)pseudouracil, 5-(methyl)-2,4-(dithio)pseudouracil, 1-substituted pseudouracil, 1-substituted 2(thio)-pseudouracil, 1-substituted 4-(thio)pseudouracil, 1-substituted 2,4-(dithio)pseudouracil, 1-(aminocarbonylethylenyl)-pseudouracil, 1-(aminocarbonylethylenyl)-2(thio)-pseudouracil, 1-(aminocarbonylethylenyl)-4-(thio)pseudouracil, 1-(aminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1-(aminoalkylaminocarbonylethylenyl)-pseudouracil, 1-(aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil, 1-(aminoalkylaminocarbonylethylenyl)-4-(thio)pseudouracil, 1-(aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-substituted 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-substituted 1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(guanidiniumalkyl-

hydroxy)-1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 1,3,5-(triaza)-2,6-(dioxa)-naphthalene, inosine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, inosinyl, 2-aza-inosinyl, 7-deaza-inosinyl, nitroimidazolyl, nitropyrazolyl, nitrobenzimidazolyl, nitroindazolyl, aminoindolyl, pyrrolopyrimidinyl, 3-(methyl)isocarbostyrilyl, 5-(methyl)isocarbostyrilyl, 3-(methyl)-7-(propynyl)isocarbostyrilyl, 7-(aza)indolyl, 6-(methyl)-7-(aza)indolyl, imidizopyridinyl, 9-(methyl)-imidizopyridinyl, pyrrolopyrizinyl, isocarbostyrilyl, 7-(propynyl)isocarbostyrilyl, propynyl-7-(aza)indolyl, 2,4,5-(trimethyl)phenyl, 4-(methyl)indolyl, 4,6-(dimethyl)indolyl, phenyl, napthalenyl, anthracenyl, phenanthracenyl, pyrenyl, stilbenyl, tetracenyl, pentacenyl, difluorotolyl, 4-(fluoro)-6-(methyl)benzimidazole, 4-(methyl)benzimidazole, 6-(azo)thymine, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 6-(aza)pyrimidine, 2-(amino)purine, 2,6-(diamino)purine, 5-substituted pyrimidines, N^2-substituted purines, N^6-substituted purines, O^6-substituted purines, substituted 1,2,4-triazoles, pyrrolo-pyrimidin-2-on-3-yl, 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, *para*-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, *ortho*-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-*ortho*-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, *para*-(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, *ortho*-(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-*ortho*--(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, pyridopyrimidin-3-yl, 2-oxo-7-amino-pyridopyrimidin-3-yl, 2-oxo-pyridopyrimidine-3-yl, or any O-alkylated or N-alkylated derivatives thereof. Alternatively, substituted or modified analogs of any of the above bases and "universal bases" can be employed.

[0276] As used herein, a universal nucleobase is any nucleobase that can base pair with all of the four naturally occurring nucleobases without substantially affecting the melting behavior, recognition by intracellular enzymes or activity of the iRNA duplex. Some exemplary universal nucleobases include, but are not limited to, 2,4-difluorotoluene, nitropyrrolyl, nitroindolyl, 8-aza-7-deazaadenine, 4-fluoro-6-methylbenzimidazle, 4-methylbenzimidazle, 3-methyl isocarbostyrilyl, 5- methyl isocarbostyrilyl, 3-methyl-7-propynyl isocarbostyrilyl, 7-azaindolyl, 6-methyl-7-azaindolyl, imidizopyridinyl, 9-methyl-imidizopyridinyl, pyrrolopyrizinyl, isocarbostyrilyl, 7-propynyl isocarbostyrilyl, propynyl-7-azaindolyl, 2,4,5-trimethylphenyl, 4-methylinolyl, 4,6-dimethylindolyl, phenyl, napthalenyl, anthracenyl, phenanthracenyl, pyrenyl, stilbenyl, tetracenyl, pentacenyl, and structural derivatives thereof (see for example, Loakes, 2001, *Nucleic Acids Research*, 29, 2437-2447).

[0277] Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808; those

disclosed in International Application No. PCT/US09/038425, filed March 26, 2009; those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990; those disclosed by English *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613; those disclosed in Modified Nucleosides in Biochemistry, Biotechnology and Medicine, Herdewijin, P.Ed. Wiley-VCH, 2008; and those disclosed by Sanghvi, Y.S., Chapter 15, dsRNA Research and Applications, pages 289-302, Crooke, S.T. and Lebleu, B., Eds., CRC Press, 1993. Contents of all of the above are herein incorporated by reference.

[0278] In certain embodiments, a modified nucleobase is a nucleobase that is fairly similar in structure to the parent nucleobase, such as for example a 7-deaza purine, a 5-methyl cytosine, or a G-clamp. In certain embodiments, nucleobase mimetic includes more complicated structures, such as for example a tricyclic phenoxazine nucleobase mimetic. Methods for preparation of the above noted modified nucleobases are well known to those skilled in the art.

Nucleic acid modifications (sugar)

[0279] The oligonucleotide provided herein can comprise one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) monomer, including a nucleoside or nucleotide, having a modified sugar moiety. For example, the furanosyl sugar ring of a nucleoside can be modified in a number of ways including, but not limited to, addition of a substituent group, bridging of two non-geminal ring atoms to form a locked nucleic acid or bicyclic nucleic acid. In certain embodiments, oligonucleotides comprise one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) monomers that are LNA.

[0280] In some embodiments of a locked nucleic acid, the 2′ position of furnaosyl is connected to the 4’ position by a linker selected independently from –$[C(R1)(R2)]_n$–, –$[C(R1)(R2)]_n$–O–, –$[C(R1)(R2)]_n$-N(R1)–, –$[C(R1)(R2)]_n$-N(R1)–O-, —$[C(R1R2)]_n$-O-N(R1)—, –C(R1)=C(R2)–O–, –C(R1)=N–, –C(R1)=N–O-, —C(=NR1)-, —C(=NR1)-O-, —C(=O)—, —C(=O)O—, —C(=S)—, —C(=S)O—, —C(=S)S—, —O—, —Si(R1)2-, —$S(=O)_x$- and —N(R1)-;

wherein:

x is 0, 1, or 2;

n is 1, 2, 3, or 4;

each R1 and R2 is, independently, H, a protecting group, hydroxyl, C1-C12 alkyl,

substituted C1-C12 alkyl, C2-C12 alkenyl, substituted C2-C12 alkenyl, C2-C12 alkynyl, substituted C2-C12 alkynyl, C5-C20 aryl, substituted C5-C20 aryl, heterocycle radical, substituted heterocycle radical, heteroaryl, substituted heteroaryl, C5-C7 alicyclic radical, substituted C5-C7 alicyclic radical, halogen, OJ1, NJ1J2, SJ1, N3, COOJ1, acyl (C(=O)—H), substituted acyl, CN, sulfonyl (S(=O)2-J1), or sulfoxyl (S(=O)-J1); and

each J1 and J2 is, independently, H, C1-C12 alkyl, substituted C1-C12 alkyl, C2-C12 alkenyl, substituted C2-C12 alkenyl, C2-C12 alkynyl, substituted C2-C12 alkynyl, C5-C20 aryl, substituted C5-C20 aryl, acyl (C(=O)—H), substituted acyl, a heterocycle radical, a substituted heterocycle radical, C1-C12 aminoalkyl, substituted C1-C12 aminoalkyl or a protecting group.

[0281] In some embodiments, each of the linkers of the LNA compounds is, independently, —[C(R1)(R2)]n-, —[C(R1)(R2)]n-O—, —C(R1R2)-N(R1)-O— or —C(R1R2)-O—N(R1)-. In another embodiment, each of said linkers is, independently, 4′-CH_2-2′, 4′-$(CH_2)_2$-2′, 4′-$(CH_2)_3$-2′, 4′-CH_2-O-2′, 4′-$(CH_2)_2$-O-2′, 4′-CH_2-O—N(R1)-2′ and 4′-CH_2-N(R1)-O-2′- wherein each R1 is, independently, H, a protecting group or C1-C12 alkyl.

[0282] Certain LNA's have been prepared and disclosed in the patent literature as well as in scientific literature (Singh et al., Chem. Commun., 1998, 4, 455-456; Koshkin et al., Tetrahedron, 1998, 54, 3607-3630; Wahlestedt et al., Proc. Natl. Acad. Sci. U.S.A., 2000, 97, 5633-5638; Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222; WO 94/14226; WO 2005/021570; Singh et al., J. Org. Chem., 1998, 63, 10035-10039; Examples of issued US patents and published applications that disclose LNA s include, for example, U.S. Pat. Nos. 7,053,207; 6,268,490; 6,770,748; 6,794,499; 7,034,133; and 6,525,191; and U.S. Pre-Grant Publication Nos. 2004-0171570; 2004-0219565; 2004-0014959; 2003-0207841; 2004-0143114; and 20030082807.

[0283] Also provided herein are LNAs in which the 2′-hydroxyl group of the ribosyl sugar ring is linked to the 4′ carbon atom of the sugar ring thereby forming a methyleneoxy (4′-CH_2-O-2′) linkage to form the bicyclic sugar moiety (reviewed in Elayadi et al., Curr. Opinion Invens. Drugs, 2001, 2, 558-561; Braasch et al., Chem. Biol., 2001, 8 1-7; and Orum et al., Curr. Opinion Mol. Ther., 2001, 3, 239-243; see also U.S. Pat. Nos. 6,268,490 and 6,670,461). The linkage can be a methylene (—CH_2-) group bridging the 2′ oxygen atom and the 4′ carbon atom, for which the term methyleneoxy (4′-CH_2-O-2′) LNA is used for the bicyclic moiety; in the case of an ethylene group in this position, the term ethyleneoxy (4′-CH_2CH_2-O-2′) LNA is used (Singh et al., Chem. Commun., 1998, 4, 455-456: Morita et al.,

Bioorganic Medicinal Chemistry, 2003, 11, 2211-2226). Methyleneoxy (4′-CH_2-O-2′) LNA and other bicyclic sugar analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm=+3 to +10° C.), stability towards 3′-exonucleolytic degradation and good solubility properties. Potent and nontoxic antisense oligonucleotides comprising BNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U.S.A., 2000, 97, 5633-5638).

[0284] An isomer of methyleneoxy (4′-CH_2-O-2′) LNA that has also been discussed is alpha-L-methyleneoxy (4′-CH_2-O-2′) LNA which has been shown to have superior stability against a 3′-exonuclease. The alpha-L-methyleneoxy (4′-CH_2-O-2′) LNA's were incorporated into antisense gapmers and chimeras that showed potent antisense activity (Frieden et al., Nucleic Acids Research, 2003, 21, 6365-6372).

[0285] The synthesis and preparation of the methyleneoxy (4′-CH_2-O-2′) LNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). BNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

[0286] Analogs of methyleneoxy (4′-CH_2-O-2′) LNA, phosphorothioate-methyleneoxy (4′-CH_2-O-2′) LNA and 2′-thio-LNAs, have also been prepared (Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs comprising oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., WO 99/14226). Furthermore, synthesis of 2′-amino-LNA, a novel comformationally restricted high-affinity oligonucleotide analog has been described in the art (Singh et al., J. Org. Chem., 1998, 63, 10035-10039). In addition, 2′-Amino- and 2′-methylamino-LNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

[0287] Modified sugar moieties are well known and can be used to alter, typically increase, the affinity of the antisense compound for its target and/or increase nuclease resistance. A representative list of preferred modified sugars includes but is not limited to bicyclic modified sugars, including methyleneoxy (4′-CH_2-O-2′) LNA and ethyleneoxy (4′-$(CH_2)_2$-O-2′ bridge) ENA; substituted sugars, especially 2′-substituted sugars having a 2′-F, 2′-OCH_3 or a 2′-$O(CH_2)_2$-OCH_3 substituent group; and 4′-thio modified sugars. Sugars can also be replaced with sugar mimetic groups among others. Methods for the preparations of modified sugars are well known to those skilled in the art. Some representative patents and

publications that teach the preparation of such modified sugars include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; 5,700,920; 6,531,584; and 6,600,032; and WO 2005/121371.

[0288] Examples of "oxy"-2′ hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$, n =1-50; "locked" nucleic acids (LNA) in which the furanose portion of the nucleoside includes a bridge connecting two carbon atoms on the furanose ring, thereby forming a bicyclic ring system; O-AMINE or $O-(CH_2)_nAMINE$ (n = 1-10, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, ethylene diamine or polyamino); and O-$CH_2CH_2(NCH_2CH_2NMe_2)_2$.

[0289] "Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars, which are of particular relevance to the single-strand overhangs); halo (*e.g.*, fluoro); amino (*e.g.* NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino); -NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; thioalkyl; alkyl; cycloalkyl; aryl; alkenyl and alkynyl, which can be optionally substituted with *e.g.*, an amino functionality.

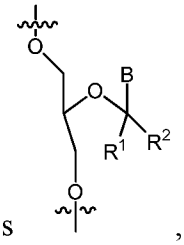
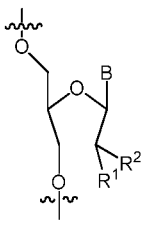
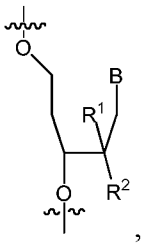
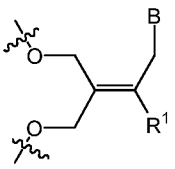
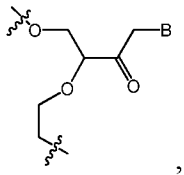
[0290] Other suitable 2'-modifications, e.g., modified MOE, are described in U.S. Patent Application Publication No. 20130130378, contents of which are herein incorporated by reference.

[0291] A modification at the 2' position can be present in the arabinose configuration The term "arabinose configuration" refers to the placement of a substituent on the C2' of ribose in the same configuration as the 2'-OH is in the arabinose.

[0292] The sugar can comprise two different modifications at the same carbon in the sugar, e.g., *gem* modification. The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, an oligonucleotide can include one or more monomers containing *e.g.*, arabinose, as the sugar. The monomer can have an alpha linkage at the 1' position on the sugar, e.g., alpha-nucleosides. The monomer can also have the opposite configuration at the

4'-position, e.g., C5' and H4' or substituents replacing them are interchanged with each other. When the C5' and H4' or substituents replacing them are interchanged with each other, the sugar is said to be modified at the 4' position.

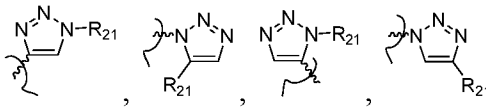
[0293] The oligonucleotide disclosed herein can also include abasic sugars, *i.e.*, a sugar which lack a nucleobase at C-1′ or has other chemical groups in place of a nucleobase at C1'. See for example U.S. Pat. No. 5,998,203, content of which is herein incorporated in its entirety. These abasic sugars can also be further containing modifications at one or more of the constituent sugar atoms. The oligonucleotide can also contain one or more sugars that are the L isomer, e.g. L-nucleosides. Modification to the sugar group can also include replacement of the 4'-O with a sulfur, optionally substituted nitrogen or CH_2 group. In some embodiments, linkage between C1' and nucleobase is in α configuration.

[0294] Sugar modifications can also include a "acyclic nucleotide," which refers to any nucleotide having an acyclic ribose sugar, e.g., wherein a C-C bonds between ribose carbons (e.g., C1'-C2', C2'-C3', C3'-C4', C4'-O4', C1'-O4') is absent and/or at least one of ribose carbons or oxygen (e.g., C1', C2', C3', C4' or O4') are independently or in combination absent from the nucleotide. In some embodiments, acyclic nucleotide is  ,  ,  ,  or  , wherein B is a modified or unmodified nucleobase, R_1 and R_2 independently are H, halogen, OR_3, or alkyl; and R_3 is H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar).

[0295] In some embodiments, sugar modifications are selected from the group consisting of 2'-H, 2′-*O*-Me (2′-*O*-methyl), 2′-*O*-MOE (2′-*O*-methoxyethyl), 2'-F, 2′-*O*-[2-(methylamino)-2-oxoethyl] (2′-*O*-NMA), 2'-*S*-methyl, 2'-O-CH_2-(4'-C) (LNA), 2'-O-CH_2CH_2-(4'-C) (ENA), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE) and *gem* 2'-OMe/2'F with 2'-O-Me in the arabinose configuration.

[0296] It is to be understood that when a particular nucleotide is linked through its 2'-

position to the next nucleotide, the sugar modifications described herein can be placed at the 3'-position of the sugar for that particular nucleotide, e.g., the nucleotide that is linked through its 2' -position. A modification at the 3' position can be present in the xylose configuration The term "xylose configuration" refers to the placement of a substituent on the C3' of ribose in the same configuration as the 3'-OH is in the xylose sugar.

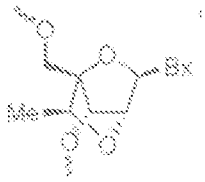
[0297] The hydrogen attached to C4' and/or C1' can be replaced by a straight- or branched- optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, wherein backbone of the alkyl, alkenyl and alkynyl can contain one or more of O, S, S(O), SO_2, N(R'), C(O), N(R')C(O)O, OC(O)N(R'), CH(Z'), phosphorous containing linkage, optionally substituted aryl, optionally substituted heteroaryl, optionally substituted heterocyclic or optionally substituted cycloalkyl, where R' is hydrogen, acyl or optionally substituted aliphatic, Z' is selected from the group consisting of OR_{11}, COR_{11}, CO_2R_{11},  , $NR_{21}R_{31}$, $CONR_{21}R_{31}$, $CON(H)NR_{21}R_{31}$, $ONR_{21}R_{31}$, $CON(H)N=CR_{41}R_{51}$, $N(R_{21})C(=NR_{31})NR_{21}R_{31}$, $N(R_{21})C(O)NR_{21}R_{31}$, $N(R_{21})C(S)NR_{21}R_{31}$, $OC(O)NR_{21}R_{31}$, $SC(O)NR_{21}R_{31}$, $N(R_{21})C(S)OR_{11}$, $N(R_{21})C(O)OR_{11}$, $N(R_{21})C(O)SR_{11}$, $N(R_{21})N=CR_{41}R_{51}$, $ON=CR_{41}R_{51}$, SO_2R_{11}, SOR_{11}, SR_{11}, and substituted or unsubstituted heterocyclic; R_{21} and R_{31} for each occurrence are independently hydrogen, acyl, unsubstituted or substituted aliphatic, aryl, heteroaryl, heterocyclic, OR_{11}, COR_{11}, CO_2R_{11}, or $NR_{11}R_{11}$'; or R_{21} and R_{31}, taken together with the atoms to which they are attached, form a heterocyclic ring; R_{41} and R_{51} for each occurrence are independently hydrogen, acyl, unsubstituted or substituted aliphatic, aryl, heteroaryl, heterocyclic, OR_{11}, COR_{11}, or CO_2R_{11}, or $NR_{11}R_{11}$'; and R_{11} and R_{11}' are independently hydrogen, aliphatic, substituted aliphatic, aryl, heteroaryl, or heterocyclic. In some embodiments, the hydrogen attached to the C4' of the 5' terminal nucleotide is replaced.

[0298] In some embodiments, C4' and C5' together form an optionally substituted heterocyclic, preferably comprising at least one -PX(Y)-, wherein X is H, OH, OM, SH, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkylthio, optionally substituted alkylamino or optionally substituted dialkylamino, where M is independently for each occurrence an alkali metal or transition metal with an overall charge of +1; and Y is O, S, or NR', where R' is hydrogen, optionally substituted aliphatic. Preferably this modification is at the 5' terminal of the iRNA.

[0299] In certain embodiments, the oligonucleotide comprises at least two regions of at

least two contiguous monomers of the above formula. In certain embodiments, the oligonucleotide comprises a gapped motif. In certain embodiments, the oligonucleotide comprises at least one region of from about 8 to about 14 contiguous β-D-2′-deoxyribofuranosyl nucleosides. In certain embodiments, the oligonucleotide comprises at least one region of from about 9 to about 12 contiguous β-D-2′-deoxyribofuranosyl nucleosides.

[0300] In certain embodiments, the oligonucleotide comprises at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more) comprises at least one (S)-cEt monomer of the formula:



S-cEt (C) ,

wherein Bx is heterocyclic base moiety.

[0301] In certain embodiments, monomers include sugar mimetics. In certain such embodiments, a mimetic is used in place of the sugar or sugar-internucleoside linkage combination, and the nucleobase is maintained for hybridization to a selected target. Representative examples of a sugar mimetics include, but are not limited to, cyclohexenyl or morpholino. Representative examples of a mimetic for a sugar-internucleoside linkage combination include, but are not limited to, peptide nucleic acids (PNA) and morpholino groups linked by uncharged achiral linkages. In some instances a mimetic is used in place of the nucleobase. Representative nucleobase mimetics are well known in the art and include, but are not limited to, tricyclic phenoxazine analogs and universal bases (Berger et al., Nuc Acid Res. 2000, 28:2911-14, incorporated herein by reference). Methods of synthesis of sugar, nucleoside and nucleobase mimetics are well known to those skilled in the art.

Nucleic acid modifications (intersugar linkage)

[0302] Described herein are linking groups that link monomers (including, but not limited to, modified and unmodified nucleosides and nucleotides) together, thereby forming an oligonucleotide. Such linking groups are also referred to as intersugar linkage. The two main classes of linking groups are defined by the presence or absence of a phosphorus atom. Representative phosphorus containing linkages include, but are not limited to, phosphodiesters (P=O), phosphotriesters, methylphosphonates, phosphoramidate, and

phosphorothioates (P=S). Representative non-phosphorus containing linking groups include, but are not limited to, methylenemethylimino (—CH_2-N(CH_3)-O—CH2-), thiodiester (—O—C(O)—S—), thionocarbamate (—O—C(O)(NH)—S—); siloxane (—O—$Si(H)_2$-O—); and N,N′-dimethylhydrazine (—CH_2-N(CH_3)-N(CH_3)-).

[0303] As discussed above, described herein is the cyclic disulfide moiety that is introduced to one or more of the phosphorous-containing internucleotide linkage groups of an oligonucleotide as a temporary protecting group. The remaining phosphorous-containing internucleotide linkage groups can also be modified using the methods described below.

[0304] Modified linkages, compared to natural phosphodiester linkages, can be used to alter, typically increase, nuclease resistance of the oligonucleotides. In certain embodiments, linkages having a chiral atom can be prepared as racemic mixtures, as separate enantiomers. Representative chiral linkages include, but are not limited to, alkylphosphonates and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known to those skilled in the art.

[0305] The phosphate group in the linking group can be modified by replacing one of the oxygens with a different substituent. One result of this modification can be increased resistance of the oligonucleotide to nucleolytic breakdown. Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. In some embodiments, one of the non-bridging phosphate oxygen atoms in the linkage can be replaced by any of the following: S, Se, BR_3 (R is hydrogen, alkyl, aryl), C (i.e. an alkyl group, an aryl group, etc…), H, NR_2 (R is hydrogen, optionally substituted alkyl, aryl), or (R is optionally substituted alkyl or aryl). The phosphorous atom in an unmodified phosphate group is achiral. However, replacement of one of the non-bridging oxygens with one of the above atoms or groups of atoms renders the phosphorous atom chiral; in other words a phosphorous atom in a phosphate group modified in this way is a stereogenic center. The stereogenic phosphorous atom can possess either the “R” configuration (herein Rp) or the “S” configuration (herein Sp).

[0306] Phosphorodithioates have both non-bridging oxygens replaced by sulfur. The phosphorus center in the phosphorodithioates is achiral which precludes the formation of oligonucleotides diastereomers. Thus, while not wishing to be bound by theory, modifications to both non-bridging oxygens, which eliminate the chiral center, *e.g.* phosphorodithioate formation, can be desirable in that they cannot produce diastereomer

mixtures. Thus, the non-bridging oxygens can be independently any one of O, S, Se, B, C, H, N, or OR (R is alkyl or aryl).

[0307] The phosphate linker can also be modified by replacement of bridging oxygen, (i.e. oxygen that links the phosphate to the sugar of the monomer), with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at the either one of the linking oxygens or at both linking oxygens. When the bridging oxygen is the 3'-oxygen of a nucleoside, replacement with carbon is preferred. When the bridging oxygen is the 5'-oxygen of a nucleoside, replacement with nitrogen is preferred.

[0308] Modified phosphate linkages where at least one of the oxygen linked to the phosphate has been replaced or the phosphate group has been replaced by a non-phosphorous group, are also referred to as "non-phosphodiester intersugar linkage" or "non-phosphodiester linker."

[0309] In certain embodiments, the phosphate group can be replaced by non-phosphorus containing connectors, e.g. dephospho linkers. Dephospho linkers are also referred to as non-phosphodiester linkers herein. While not wishing to be bound by theory, it is believed that since the charged phosphodiester group is the reaction center in nucleolytic degradation, its replacement with neutral structural mimics should impart enhanced nuclease stability. Again, while not wishing to be bound by theory, it can be desirable, in some embodiment, to introduce alterations in which the charged phosphate group is replaced by a neutral moiety.

[0310] Examples of moieties which can replace the phosphate group include, but are not limited to, amides (for example amide-3 (3'-CH_2-C(=O)-N(H)-5') and amide-4 (3'-CH_2-N(H)-C(=O)-5')), hydroxylamino, siloxane (dialkylsiloxane), carboxamide, carbonate, carboxymethyl, carbamate, carboxylate ester, thioether, ethylene oxide linker, sulfide, sulfonate, sulfonamide, sulfonate ester, thioformacetal (3'-S-CH_2-O-5'), formacetal (3 '-O-CH_2-O-5'), oxime, methyleneimino, methykenecarbonylamino, methylenemethylimino (MMI, 3'-CH_2-N(CH_3)-O-5'), methylenehydrazo, methylenedimethylhydrazo, methyleneoxymethylimino, ethers (C3'-O-C5'), thioethers (C3'-S-C5'), thioacetamido (C3'-N(H)-C(=O)-CH_2-S-C5', C3'-O-P(O)-O-SS-C5', C3'-CH_2-NH-NH-C5', 3'-NHP(O)(OCH_3)-O-5' and 3'-NHP(O)(OCH_3)-O-5' and nonionic linkages containing mixed N, O, S and CH_2 component parts. See for example, Carbohydrate Modifications in Antisense Research; Y.S. Sanghvi and P.D. Cook Eds. ACS Symposium Series 580; Chapters 3 and 4, (pp. 40-65). Preferred embodiments include methylenemethylimino (MMI), methylenecarbonylamino,

amides, carbamate and ethylene oxide linker.

[0311] One skilled in the art is well aware that in certain instances replacement of a non-bridging oxygen can lead to enhanced cleavage of the intersugar linkage by the neighboring 2'-OH, thus in many instances, a modification of a non-bridging oxygen can necessitate modification of 2'-OH, e.g., a modification that does not participate in cleavage of the neighboring intersugar linkage, e.g., arabinose sugar, 2'-O-alkyl, 2'-F, LNA and ENA.

[0312] Preferred non-phosphodiester intersugar linkages include phosphorothioates, phosphorothioates with an at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% , 90% 95% or more enantiomeric excess of Sp isomer, phosphorothioates with an at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% , 90% 95% or more enantiomeric excess of Rp isomer, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, alkyl-phosphonaters (e.g., methyl-phosphonate), selenophosphates, phosphoramidates (e.g., N-alkylphosphoramidate), and boranophosphonates.

[0313] In some embodiments, the oligonucleotide further comprises at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more and up to including all) modified or nonphosphodiester linkages. In some embodiments, the oligonucleotide further comprises at least one (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more and up to including all) phosphorothioate linkages.

[0314] The oligonucleotide can also be constructed wherein the phosphate linker and the sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates. While not wishing to be bound by theory, it is believed that the absence of a repetitively charged backbone diminishes binding to proteins that recognize polyanions (*e.g.* nucleases). Again, while not wishing to be bound by theory, it can be desirable in some embodiment, to introduce alterations in which the bases are tethered by a neutral surrogate backbone. Examples include the morpholino, cyclobutyl, pyrrolidine, peptide nucleic acid (PNA), aminoethylglycyl PNA (*aeg*PNA) and backbone-extended pyrrolidine PNA (*bep*PNA) nucleoside surrogates. A preferred surrogate is a PNA surrogate.

[0315] The oligonucleotide described herein can contain one or more asymmetric centers and thus give rise to enantiomers, diastereomers, and other stereoisomeric configurations that may be defined, in terms of absolute stereochemistry, as (R) or (S), such as for sugar anomers, or as (D) or (L) such as for amino acids et al. Included in the oligonucleotide are all such possible isomers, as well as their racemic and optically pure forms.

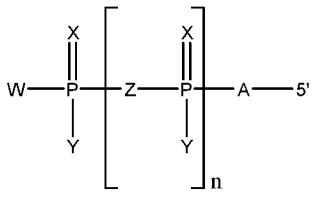
Nucleic acid modifications (terminal modifications)

[0316] In some embodiments, the oligonucleotide further comprises a phosphate or phosphate mimic at the 5'-end of the antisense strand. In one embodiment, the phosphate mimic is a 5'-vinyl phosphonate (VP).

[0317] In some embodiments, the 5'-end of the antisense strand does not contain a 5'-vinyl phosphonate (VP).

[0318] Ends of the iRNA agent can be modified. Such modifications can be at one end or both ends. For example, the 3′ and/or 5′ ends of an iRNA can be conjugated to other functional molecular entities such as labeling moieties, *e.g.*, fluorophores (*e.g.*, pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes) or protecting groups (based *e.g.*, on sulfur, silicon, boron or ester). The functional molecular entities can be attached to the sugar through a phosphate group and/or a linker. The terminal atom of the linker can connect to or replace the linking atom of the phosphate group or the C-3′ or C-5′ O, N, S or C group of the sugar. Alternatively, the linker can connect to or replace the terminal atom of a nucleotide surrogate (*e.g.*, PNAs).

[0319] When a linker/phosphate-functional molecular entity-linker/phosphate array is interposed between two strands of a double stranded oligonucleotide, this array can substitute for a hairpin loop in a hairpin-type oligonucleotide.

[0320] Terminal modifications useful for modulating activity include modification of the 5' end of iRNAs with phosphate or phosphate analogs. In certain embodiments, the 5'end of an iRNA is phosphorylated or includes a phosphoryl analog. Exemplary 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Modifications at the 5'-terminal end can also be useful in stimulating or inhibiting the immune system of a subject. In some embodiments, the 5'-end of the oligonucleotide comprises the modification  , wherein W, X and Y are each independently selected from the group consisting of O, OR (R is hydrogen, alkyl, aryl), S, Se, BR_3 (R is hydrogen, alkyl, aryl), BH_3^-, C (i.e. an alkyl group, an aryl group, etc...), H, NR_2 (R is hydrogen, alkyl, aryl), or OR (R is hydrogen, alkyl or aryl); A and Z are each independently for each occurrence absent, O, S, CH_2, NR (R is hydrogen, alkyl, aryl), or optionally substituted alkylene, wherein backbone of the alkylene can comprise one or more

of O, S, SS and NR (R is hydrogen, alkyl, aryl) internally and/or at the end; and n is 0-2. In some embodiments, n is 1 or 2. It is understood that A is replacing the oxygen linked to 5' carbon of sugar. When n is 0, W and Y together with the P to which they are attached can form an optionally substituted 5-8 membered heterocyclic, wherein W an Y are each independently O, S, NR' or alkylene. Preferably the heterocyclic is substituted with an aryl or heteroaryl. In some embodiments, one or both hydrogen on C5' of the 5'- terminal nucleotides are replaced with a halogen, e.g., F.

[0321] Exemplary 5'-modifications include, but are not limited to, 5'-monophosphate ($(HO)_2(O)P$-O-5'); 5'-diphosphate ($(HO)_2(O)P$-O-P(HO)(O)-O-5'); 5'-triphosphate ($(HO)_2(O)P$-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)2(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)2(O)P-S-5'); 5'-alpha-thiotriphosphate; 5'-beta-thiotriphosphate; 5'-gamma-thiotriphosphate; 5'-phosphoramidates ($(HO)_2(O)P$-NH-5', $(HO)(NH_2)(O)P$-O-5'). Other 5'-modification include 5'-alkylphosphonates (R(OH)(O)P-O-5', R=alkyl, e.g., methyl, ethyl, isopropyl, propyl, etc…), 5'-alkyletherphosphonates (R(OH)(O)P-O-5', R=alkylether, e.g., methoxymethyl (CH_2OMe), ethoxymethyl, etc…); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'). Other exemplary 5'-modifications include where Z is optionally substituted alkyl at least once, e.g., $((HO)_2(X)P$-O[-$(CH_2)_a$-O-P(X)(OH)-O]$_b$- 5', $((HO)_2(X)P$-O[-$(CH_2)_a$-P(X)(OH)-O]$_b$- 5', ((HO)2(X)P-[-$(CH_2)_a$-O-P(X)(OH)-O]$_b$- 5'; dialkyl terminal phosphates and phosphate mimics: HO[-$(CH_2)_a$-O-P(X)(OH)-O]$_b$- 5' , H_2N[-$(CH_2)_a$-O-P(X)(OH)-O]$_b$- 5', H[-$(CH_2)_a$-O-P(X)(OH)-O]$_b$- 5', Me_2N[-$(CH_2)_a$-O-P(X)(OH)-O]$_b$- 5', HO[-$(CH_2)_a$-P(X)(OH)-O]$_b$- 5' , H_2N[-$(CH_2)_a$-P(X)(OH)-O]$_b$- 5', H[-$(CH_2)_a$-P(X)(OH)-O]$_b$- 5', Me_2N[-$(CH_2)_a$-P(X)(OH)-O]$_b$- 5', wherein a and b are each independently 1-10. Other embodiments, include replacement of oxygen and/or sulfur with BH_3, BH_3^- and/or Se.

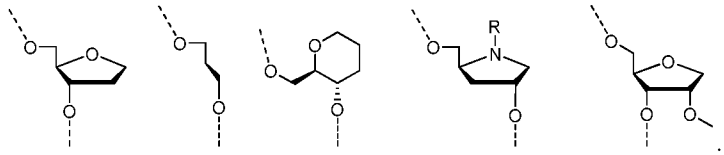
[0322] Terminal modifications can also be useful for monitoring distribution, and in such cases the preferred groups to be added include fluorophores, *e.g.*, fluorescein or an Alexa dye, *e.g.*, Alexa 488. Terminal modifications can also be useful for enhancing uptake, useful modifications for this include targeting ligands. Terminal modifications can also be useful for cross-linking an oligonucleotide to another moiety; modifications useful for this include mitomycin C, psoralen, and derivatives thereof.

Thermally Destabilizing Modifications

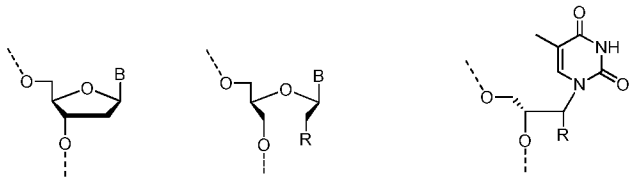
[0323] The oligonucleotide, such as iRNAs or dsRNA agents, can be optimized for RNA interference by increasing the propensity of the iRNA duplex to disassociate or melt (decreasing the free energy of duplex association) by introducing a thermally destabilizing modification in the sense strand at a site opposite to the seed region of the antisense strand (i.e., at positions 2-8 of the 5'-end of the antisense strand). This modification can increase the propensity of the duplex to disassociate or melt in the seed region of the antisense strand.

[0324] The thermally destabilizing modifications can include abasic modification; mismatch with the opposing nucleotide in the opposing strand; and sugar modification such as 2'-deoxy modification or acyclic nucleotide, e.g., unlocked nucleic acids (UNA) or glycerol nucleic acid (GNA).

[0325] Exemplified abasic modifications are:

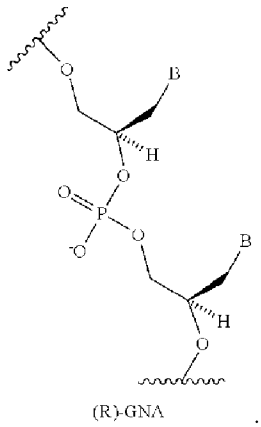


[0326] Exemplified sugar modifications are:



[0327] The term "UNA" refers to unlocked acyclic nucleic acid, wherein any of the bonds of the sugar has been removed, forming an unlocked "sugar" residue. In one example, UNA also encompasses monomers with bonds between C1'-C4' being removed (i.e. the covalent carbon-oxygen-carbon bond between the C1' and C4' carbons). In another example, the C2'-C3' bond (i.e. the covalent carbon-carbon bond between the C2' and C3' carbons) of the sugar is removed (see Mikhailov et. al., Tetrahedron Letters, 26 (17): 2059 (1985); and Fluiter et al., Mol. Biosyst., 10: 1039 (2009), which are hereby incorporated by reference in their entirety). The acyclic derivative provides greater backbone flexibility without affecting the Watson-Crick pairings. The acyclic nucleotide can be linked via 2'-5' or 3'-5' linkage.

[0328] The term 'GNA' refers to glycol nucleic acid which is a polymer similar to DNA or RNA but differing in the composition of its "backbone" in that is composed of repeating glycerol units linked by phosphodiester bonds:



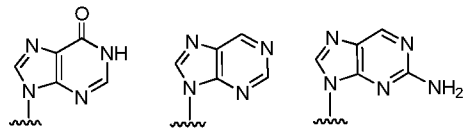
(R)-GNA

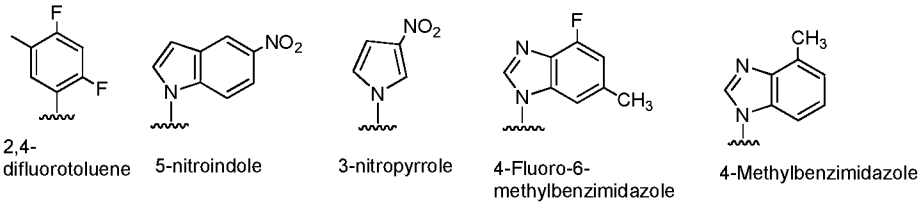
[0329] The thermally destabilizing modification can be mismatches (i.e., noncomplementary base pairs) between the thermally destabilizing nucleotide and the opposing nucleotide in the opposite strand within the dsRNA duplex. Exemplary mismatch basepairs include G:G, G:A, G:U, G:T, A:A, A:C, C:C, C:U, C:T, U:U, T:T, U:T, or a combination thereof. Other mismatch base pairings known in the art are also amenable to the present invention. A mismatch can occur between nucleotides that are either naturally occurring nucleotides or modified nucleotides, i.e., the mismatch base pairing can occur between the nucleobases from respective nucleotides independent of the modifications on the ribose sugars of the nucleotides. In certain embodiments, the oligonucleotide, such as siRNA or iRNA agent, contains at least one nucleobase in the mismatch pairing that is a 2'-deoxy nucleobase; e.g., the 2'-deoxy nucleobase is in the sense strand.

[0330] More examples of abasic nucleotide, acyclic nucleotide modifications (including UNA and GNA), and mismatch modifications have been described in detail in WO 2011/133876, which is herein incorporated by reference in its entirety.

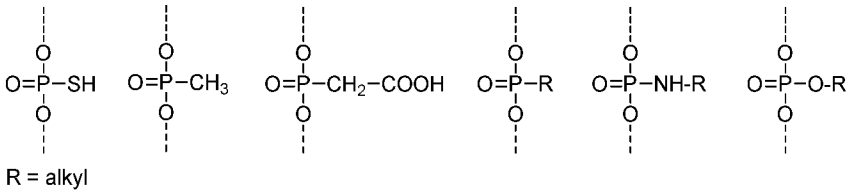
[0331] The thermally destabilizing modifications may also include universal base with reduced or abolished capability to form hydrogen bonds with the opposing bases, and phosphate modifications.

[0332] Nucleobase modifications with impaired or completely abolished capability to form hydrogen bonds with bases in the opposite strand have been evaluated for destabilization of the central region of the dsRNA duplex as described in WO 2010/0011895, which is herein incorporated by reference in its entirety. Exemplary nucleobase modifications are:





[0333] Exemplary phosphate modifications known to decrease the thermal stability of dsRNA duplexes compared to natural phosphodiester linkages are:



[0334] In some embodiments, the oligonucleotide can comprise 2'-5' linkages (with 2'-H, 2'-OH and 2'-OMe and with P=O or P=S). For example, the 2'-5' linkages modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

[0335] In another embodiment, the oligonucleotide can comprise L sugars (e.g., L ribose, L-arabinose with 2'-H, 2'-OH and 2'-OMe). For example, these L sugar modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

[0336] In some embodiments, one or more targeting ligands are connected to the modified phosphate prodrug compound via any one of R_2, R_3, R_4, R_5, R_6, R_7, R_8, and R_9 of the cyclic disulfide moiety, optionally via one or more linkers/tethers.

[0337] Introduction of the targeting ligands into an oligonucleotide via a cyclic disulfide moiety, on either the sense or antisense strand or both the sense and antisense strands, are illustrated in Scheme 16 in Example 10 below. These targeting ligands can be cleaved off with the cyclic disulfide moiety after the siRNA oligonucleotide enters into cytosol.

[0338] In some embodiments, the targeting ligand is selected from the group consisting of an antibody, a ligand-binding portion of a receptor, a ligand for a receptor, an aptamer, a

carbohydrate-based ligand, a fatty acid, a lipoprotein, folate, thyrotropin, melanotropin, surfactant protein A, mucin, glycosylated polyaminoacids, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipophilic moiety that enhances plasma protein binding, a cholesterol, a steroid, bile acid, vitamin B12, biotin, a fluorophore, and a peptide.

[0339] In certain embodiments, at least one ligand is a carbohydrate-based ligand targeting a liver tissue. In one embodiment, the carbohydrate-based ligand is selected from the group consisting of galactose, multivalent galactose, N-acetyl-galactosamine (GalNAc), multivalent GalNAc, mannose, multivalent mannose, lactose, multivalent lactose, N-acetyl-glucosamine (GlcNAc), multivalent GlcNAc, glucose, multivalent glucose, fucose, and multivalent fucose.

[0340] In certain embodiments, at least one ligand is a lipophilic moiety. In one embodiment, the lipophilicity of the lipophilic moiety, measured by $\log K_{ow}$, exceeds 0, or the hydrophobicity of the compound, measured by the unbound fraction in the plasma protein binding assay of the compound, exceeds 0.2.

[0341] In one embodiment, the lipophilic moiety contains a saturated or unsaturated C_4-C_{30} hydrocarbon chain, and an optional functional group selected from the group consisting of hydroxyl, amine, carboxylic acid, sulfonate, phosphate, thiol, azide, and alkyne. For instance, the lipophilic moiety contains a saturated or unsaturated C_6-C_{18} hydrocarbon chain.

[0342] Additional lipophilic moieties and additional details regarding lipophilicity of the lipophilic moiety and hydrophobicity of the oligonucleotide can be found in PCT Application No. PCT/US20/59399, entitled "Extrahepatic Delivery," filed on November 6, 2020, the content of which is incorporated herein by reference in its entirety.

[0343] In certain embodiments, at least one ligand targets a receptor which mediates delivery to a CNS tissue. In one embodiment, the targeting ligand is selected from the group consisting of Angiopep-2, lipoprotein receptor related protein (LRP) ligand, bEnd.3 cell binding ligand, transferrin receptor (TfR) ligand, manose receptor ligand, glucose transporter protein, and LDL receptor ligand.

[0344] In certain embodiments, at least one ligand targets a receptor which mediates delivery to an ocular tissue. In one embodiment, the targeting ligand is selected from the group consisting of trans-retinol, RGD peptide, LDL receptor ligand, and carbohydrate based ligands.

[0345] The targeting ligands can also be introduced into the oligonucleotide directly (independent (*i.e.*, not through the cyclic disulfide moiety).

[0346] In some embodiments, the oligonucleotide contains at least one targeting ligand at the 5'-end, 3'-end, and/or internal position(s) of the antisense strand.

[0347] In some embodiments, the oligonucleotide contains at least one targeting ligand at the 5'-end, 3'-end, and/or internal position(s) of the sense strand.

[0348] In some embodiments, the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end, 3'-end, and/or internal position(s) of the antisense strand, and at least one targeting ligand at the 5'-end, 3'-end, and/or internal position(s) of the sense strand.

[0349] In one embodiment, the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end of the antisense strand, and at least one targeting ligand at the 3'-end of the sense strand.

[0350] In some embodiments, one or more targeting ligands are connected to the modified phosphate prodrug compound (via the cyclic disulfide moiety) via one or more linkers/tethers, as described below.

[0351] In some embodiments, one or more targeting ligands are connected to the oligonucleotide directly (*i.e.*, not through the cyclic disulfide moiety), via one or more linkers/tethers, as described below.

Linkers/Tethers

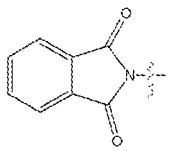
[0352] Linkers/Tethers are connected to the modified phosphate prodrug compound at a "tethering attachment point (TAP)." Linkers/Tethers may include any C_1-C_{100} carbon-containing moiety, (e.g. C_1-C_{75}, C_1-C_{50}, C_1-C_{20}, C_1-C_{10}; C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, or C_{10}), and may have at least one nitrogen atom. In certain embodiments, the nitrogen atom forms part of a terminal amino or amido (NHC(O)-) group on the linker/tether, which may serve as a connection point for the modified phosphate prodrug compound . Non-limited examples of linkers/tethers (underlined) include TAP-$(CH_2)_n$NH-; TAP-C(O)$(CH_2)_n$NH-; TAP-NR''''$(CH_2)_n$NH-, TAP-C(O)-$(CH_2)_n$-C(O)-; TAP-C(O)-$(CH_2)_n$-C(O)O-; TAP-C(O)-O-; TAP-C(O)-$(CH_2)_n$-NH-C(O)-; TAP-C(O)-$(CH_2)_n$-; TAP-C(O)-NH-; TAP-C(O)-; TAP-$(CH_2)_n$-C(O)-; TAP-$(CH_2)_n$-C(O)O-; TAP-$(CH_2)_n$-; or TAP-$(CH_2)_n$-NH-C(O)-; in which n is 1-20 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) and R'''' is C_1-C_6 alkyl. Preferably, n is 5, 6, or 11. In other embodiments, the nitrogen may form part of a terminal oxyamino group, e.g., -ONH_2, or hydrazino group, -$NHNH_2$. The linker/tether may optionally be substituted, e.g., with hydroxy, alkoxy, perhaloalkyl, and/or optionally inserted with one or more additional heteroatoms, e.g., N, O, or S. Preferred tethered ligands may

include, e.g., TAP-$(CH_2)_nNH$(LIGAND); TAP-$C(O)(CH_2)_nNH$(LIGAND); TAP-$NR''''(CH_2)_nNH$(LIGAND); TAP-$(CH_2)_nONH$(LIGAND); TAP-$C(O)(CH_2)_nONH$(LIGAND); TAP-$NR''''(CH_2)_nONH$(LIGAND); TAP-$(CH_2)_nNHNH_2$(LIGAND), TAP-$C(O)(CH_2)_nNHNH_2$(LIGAND); TAP-$NR''''(CH_2)_nNHNH_2$(LIGAND); TAP-$C(O)-(CH_2)_n-C(O)$(LIGAND); TAP-$C(O)-(CH_2)_n-C(O)O$(LIGAND); TAP-$C(O)-O$(LIGAND); TAP-$C(O)-(CH_2)_n-NH-C(O)$(LIGAND); TAP-$C(O)-(CH_2)_n$(LIGAND); TAP-$C(O)-NH$(LIGAND); TAP-$C(O)$(LIGAND); TAP-$(CH_2)_n-C(O)$ (LIGAND); TAP-$(CH_2)_n-C(O)O$(LIGAND); TAP-$(CH_2)_n$(LIGAND); or TAP-$(CH_2)_n-NH-C(O)$(LIGAND). In some embodiments, amino terminated linkers/tethers (e.g., NH_2, ONH_2, NH_2NH_2) can form an imino bond (i.e., C=N) with the ligand. In some embodiments, amino terminated linkers/tethers (e.g., NH_2, ONH_2, NH_2NH_2) can acylated, e.g., with $C(O)CF_3$.

[0353] In some embodiments, the linker/ tether can terminate with a mercapto group (i.e., SH) or an olefin (e.g., $CH=CH_2$). For example, the tether can be TAP-$(CH_2)_n-SH$, TAP-$C(O)(CH_2)_nSH$, TAP-$(CH_2)_n-(CH=CH_2)$, or TAP-$C(O)(CH_2)_n(CH=CH_2)$, in which n can be as described elsewhere. The tether may optionally be substituted, e.g., with hydroxy, alkoxy, perhaloalkyl, and/or optionally inserted with one or more additional heteroatoms, e.g., N, O, or S. The double bond can be *cis* or *trans* or *E* or *Z*.

[0354] In other embodiments, the linker/tether may include an electrophilic moiety, preferably at the terminal position of the linker/tether. Exemplary electrophilic moieties include, e.g., an aldehyde, alkyl halide, mesylate, tosylate, nosylate, or brosylate, or an activated carboxylic acid ester, e.g. an NHS ester, or a pentafluorophenyl ester. Preferred linkers/tethers (underlined) include TAP-$(CH_2)_nCHO$; TAP-$C(O)(CH_2)_nCHO$; or TAP-$NR''''(CH_2)_nCHO$, in which n is 1-6 and R'''' is C_1-C_6 alkyl; or TAP-$(CH_2)_nC(O)ONHS$; TAP-$C(O)(CH_2)_nC(O)ONHS$; or TAP-$NR''''(CH_2)_nC(O)ONHS$, in which n is 1-6 and R'''' is C_1-C_6 alkyl; TAP-$(CH_2)_nC(O)OC_6F_5$; TAP-$C(O)(CH_2)_nC(O)OC_6F_5$; or TAP-$NR''''(CH_2)_nC(O)OC_6F_5$, in which n is 1-11 and R'''' is C_1-C_6 alkyl; or -$(CH_2)_nCH_2LG$; TAP-$C(O)(CH_2)_nCH_2LG$; or TAP-$NR''''(CH_2)_nCH_2LG$, in which n can be as described elsewhere and R'''' is C_1-C_6 alkyl (LG can be a leaving group, e.g., halide, mesylate, tosylate, nosylate, brosylate). Tethering can be carried out by coupling a nucleophilic group of a ligand, e.g., a thiol or amino group with an electrophilic group on the tether.

[0355] In other embodiments, it can be desirable for the monomer to include a

phthalimido group (K) at the terminal position of the linker/tether.  K .

[0356] In other embodiments, other protected amino groups can be at the terminal position of the linker/tether, e.g., alloc, monomethoxy trityl (MMT), trifluoroacetyl, Fmoc, or aryl sulfonyl (e.g., the aryl portion can be *ortho*-nitrophenyl or *ortho, para*-dinitrophenyl).

[0357] Any of the linkers/tethers described herein may further include one or more additional linking groups, e.g., -O-$(CH_2)_n$-, -$(CH_2)_n$-SS-, -$(CH_2)_n$-, or -(CH=CH)-.

Cleavable linkers/tethers

[0358] In some embodiments, at least one of the linkers/tethers can be a redox cleavable linker, an acid cleavable linker, an esterase cleavable linker, a phosphatase cleavable linker, or a peptidase cleavable linker.

[0359] In one embodiment, at least one of the linkers/tethers can be a reductively cleavable linker (e.g., a disulfide group).

[0360] In one embodiment, at least one of the linkers/tethers can be an acid cleavable linker (e.g., a hydrazone group, an ester group, an acetal group, or a ketal group).

[0361] In one embodiment, at least one of the linkers/tethers can be an esterase cleavable linker (e.g., an ester group).

[0362] In one embodiment, at least one of the linkers/tethers can be a phosphatase cleavable linker (e.g., a phosphate group).

[0363] In one embodiment, at least one of the linkers/tethers can be a peptidase cleavable linker (e.g., a peptide bond).

[0364] Cleavable linking groups are susceptible to cleavage agents, e.g., pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

[0365] A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH at around 5.0. Some tethers will have a linkage group that is cleaved at a preferred pH, thereby releasing the iRNA agent from a ligand (e.g., a targeting or cell-permeable ligand, such as cholesterol) inside the cell, or into the desired compartment of the cell.

[0366] A chemical junction (e.g., a linking group) that links a ligand to an iRNA agent can include a disulfide bond. When the iRNA agent/ligand complex is taken up into the cell by endocytosis, the acidic environment of the endosome will cause the disulfide bond to be cleaved, thereby releasing the iRNA agent from the ligand (Quintana et al., *Pharm Res.* 19:1310-1316, 2002; Patri et al., *Curr. Opin. Curr. Biol.* 6:466-471, 2002). The ligand can be a targeting ligand or a second therapeutic agent that may complement the therapeutic effects of the iRNA agent.

[0367] A tether can include a linking group that is cleavable by a particular enzyme. The type of linking group incorporated into a tether can depend on the cell to be targeted by the iRNA agent. For example, an iRNA agent that targets an mRNA in liver cells can be conjugated to a tether that includes an ester group. Liver cells are rich in esterases, and therefore the tether will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Cleavage of the tether releases the iRNA agent from a ligand that is attached to the distal end of the tether, thereby potentially enhancing silencing activity of the iRNA agent. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

[0368] Tethers that contain peptide bonds can be conjugated to iRNA agents target to cell types rich in peptidases, such as liver cells and synoviocytes. For example, an iRNA agent targeted to synoviocytes, such as for the treatment of an inflammatory disease (e.g., rheumatoid arthritis), can be conjugated to a tether containing a peptide bond.

[0369] In general, the suitability of a candidate cleavable linking group can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood or when in contact with other non-target tissue, e.g., tissue the iRNA agent would be exposed to when administered to a subject. Thus one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be

indicative of cleavage in other tissues or biological fluids, e.g., blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It may be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least 2, 4, 10 or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood or serum (or under in vitro conditions selected to mimic extracellular conditions).

Redox Cleavable Linking Groups

[0370] One class of cleavable linking groups are redox cleavable linking groups that are cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (—S—S—). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents know in the art, which mimic the rate of cleavage which would be observed in a cell, e.g., a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In a preferred embodiment, candidate compounds are cleaved by at most 10% in the blood. In preferred embodiments, useful candidate compounds are degraded at least 2, 4, 10 or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood (or under in vitro conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

Phosphate-Based Cleavable Linking Groups

[0371] Phosphate-based linking groups are cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are —O—P(O)(ORk)-O—, —O—P(S)(ORk)-O—, —O—P(S)(SRk)-O—, —S—P(O)(ORk)-O—, —O—P(O)(ORk)-S—, —S—P(O)(ORk)-S—, —O—P(S)(ORk)-S—, —S—P(S)(ORk)-O—, —O—P(O)(Rk)-O—, —O—P(S)(Rk)-O—, —S—P(O)(Rk)-O—, —S—P(S)(Rk)-O—, —

S—P(O)(Rk)-S—, —O—P(S)(Rk)-S—. Preferred embodiments are —O—P(O)(OH)—O—, —O—P(S)(OH)—O—, —O—P(S)(SH)—O—, —S—P(O)(OH)—O—, —O—P(O)(OH)—S—, —S—P(O)(OH)—S—, —O—P(S)(OH)—S—, —S—P(S)(OH)—O—, —O—P(O)(H)—O—, —O—P(S)(H)—O—, —S—P(O)(H)—O—, —S—P(S)(H)—O—, —S—P(O)(H)—S—, —O—P(S)(H)—S—. A preferred embodiment is —O—P(O)(OH)—O—. These candidates can be evaluated using methods analogous to those described above.

Acid Cleavable Linking Groups

[0372] Acid cleavable linking groups are linking groups that are cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, ketals, acetals, esters, and esters of amino acids. Acid cleavable groups can have the general formula —C=NN—, C(O)O, or —OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

Ester-Based Linking Groups

[0373] Ester-based linking groups are cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include but are not limited to esters of alkylene, alkenylene and alkynylene groups. Ester cleavable linking groups have the general formula —C(O)O—, or —OC(O)—. These candidates can be evaluated using methods analogous to those described above.

Peptide-Based Cleaving Groups

[0374] Peptide-based linking groups are cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (e.g., dipeptides, tripeptides etc.) and polypeptides. Peptide-based cleavable groups do not include the amide group (—C(O)NH—). The amide group can be formed between any alkylene, alkenylene or alkynelene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The

peptide based cleavage group is generally limited to the peptide bond (i.e., the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide cleavable linking groups have the general formula —$NHCHR^1C(O)NHCHR^2C(O)$—, where R^1 and R^2 are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

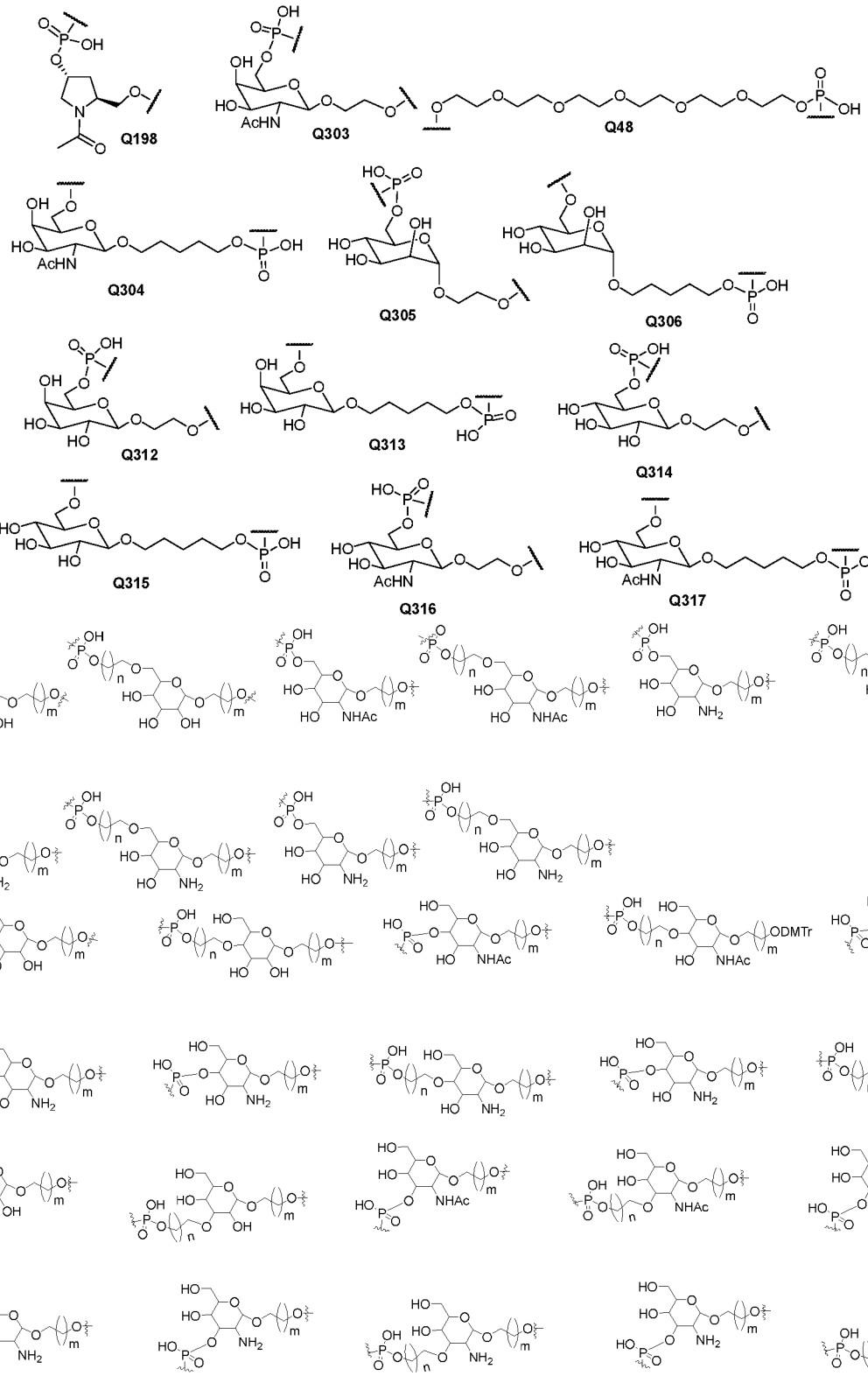
Biocleavable linkers/tethers

[0375] The linkers can also include biocleavable linkers that are nucleotide and non-nucleotide linkers or combinations thereof that connect two parts of a molecule, for example, one or both strands of two individual siRNA molecule to generate a bis(siRNA). In some embodiments, mere electrostatic or stacking interaction between two individual siRNAs can represent a linker. The non-nucleotide linkers include tethers or linkers derived from monosaccharides, disaccharides, oligosaccharides, and derivatives thereof, aliphatic, alicyclic, heterocyclic, and combinations thereof.

[0376] In some embodiments, at least one of the linkers (tethers) is a bio-cleavable linker selected from the group consisting of DNA, RNA, disulfide, amide, functionalized monosaccharides or oligosaccharides of galactosamine, glucosamine, glucose, galactose, and mannose, and combinations thereof.

[0377] In one embodiment, the bio-cleavable carbohydrate linker may have 1 to 10 saccharide units, which have at least one anomeric linkage capable of connecting two siRNA units. When two or more saccharides are present, these units can be linked via 1-3, 1-4, or 1-6 sugar linkages, or via alkyl chains.

[0378] Exemplary bio-cleavable linkers include: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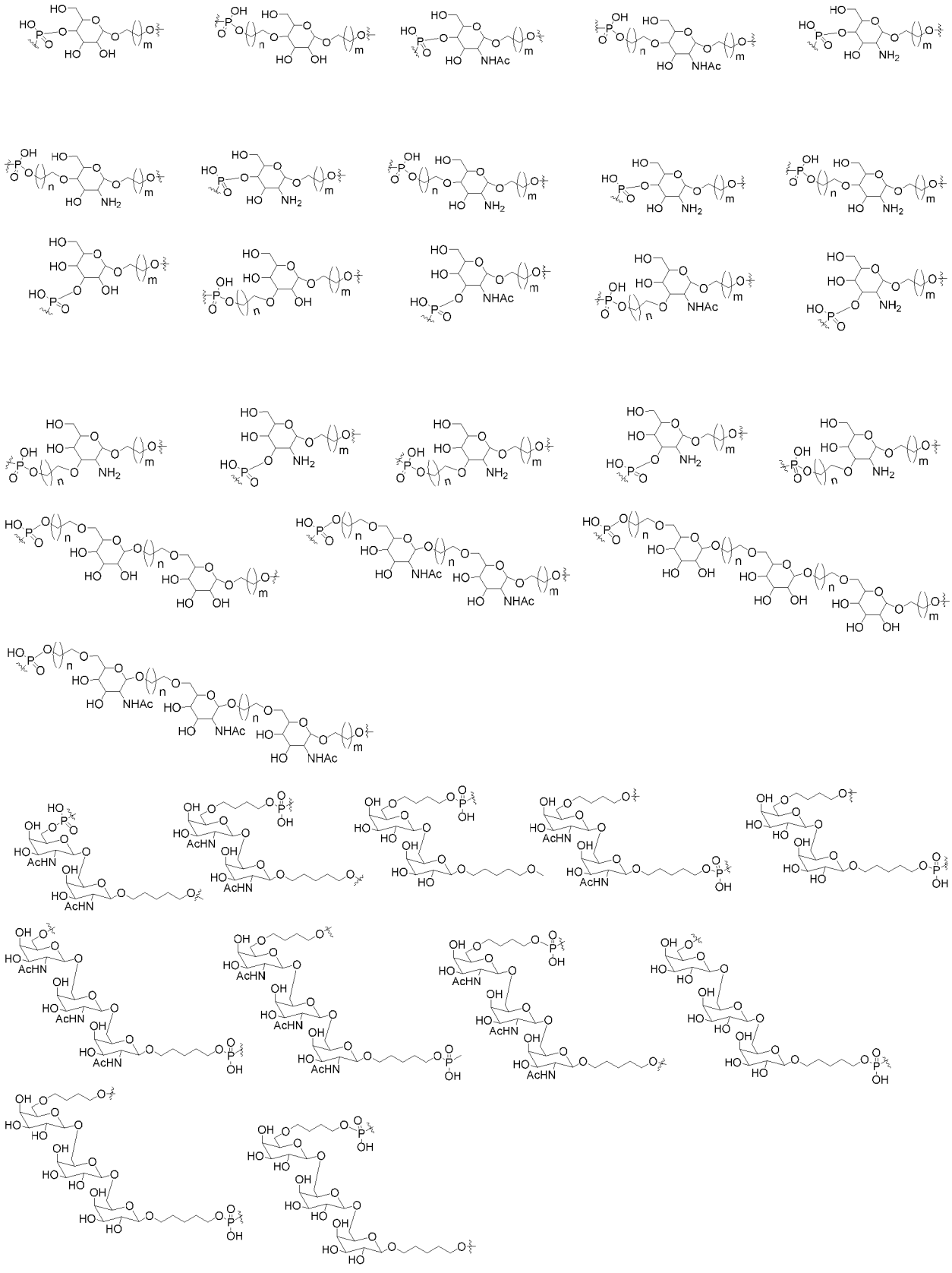
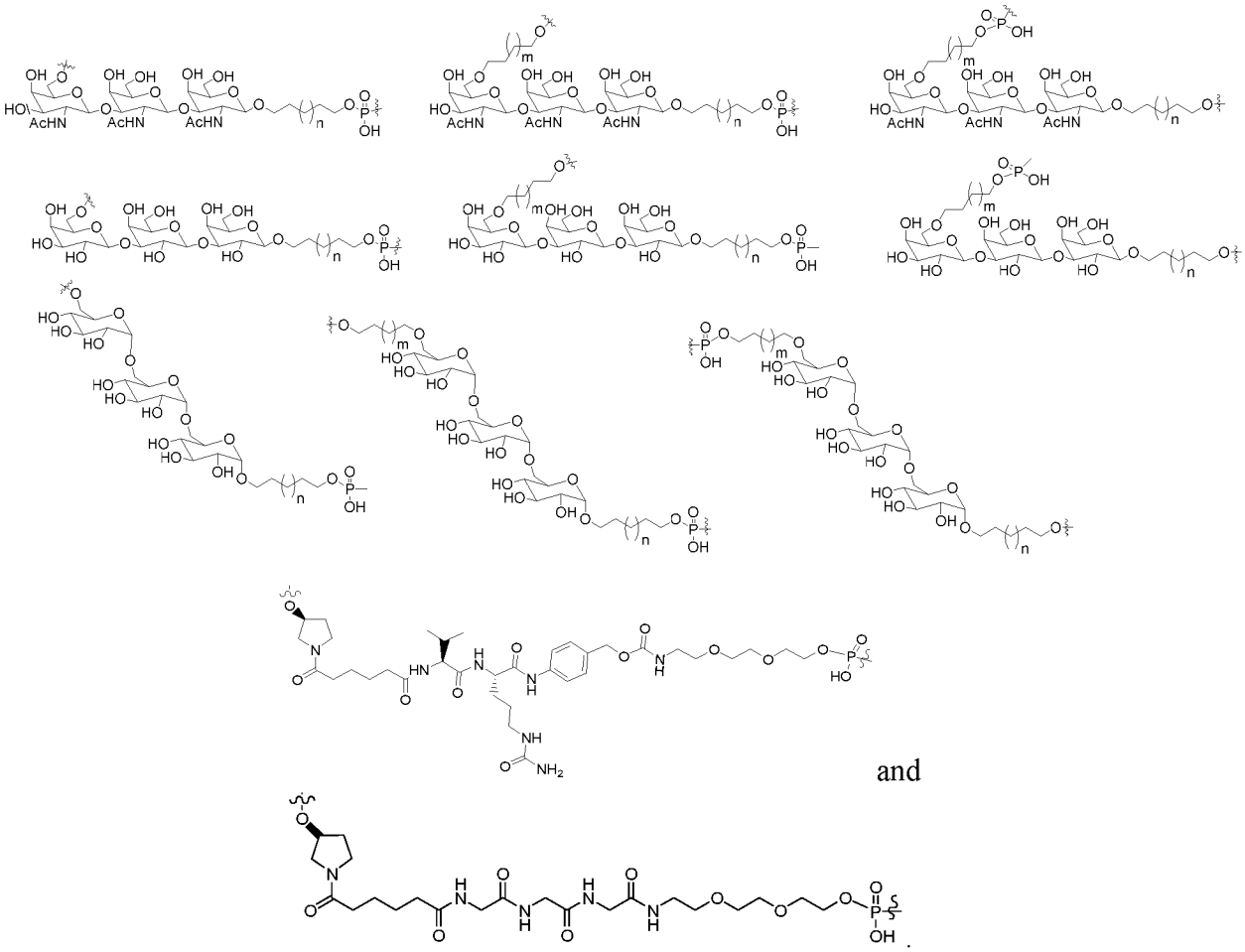




and

[0379] More discussion about the biocleavable linkers may be found in PCT application No. PCT/US18/14213, entitled "Endosomal Cleavable Linkers," filed on January 18, 2018, the content of which is incorporated herein by reference in its entirety.

Carriers

[0380] In some embodiments, one or more targeting ligands are connected to the modified phosphate prodrug compound (via the cyclic disulfide moiety) via one or more carriers, as described herein, and optionally via one or more linkers/tethers, as described above,

[0381] In some embodiments, one or more targeting ligands are connected to the oligonucleotide directly (*i.e.*, not through the cyclic disulfide moiety), via one or more carriers, as described herein, and optionally via one or more linkers/tethers, as described above.

[0382] The carrier can be a cyclic group or an acyclic group. In one embodiment, the cyclic group is selected from the group consisting of pyrrolidinyl, pyrazolinyl, pyrazolidinyl,

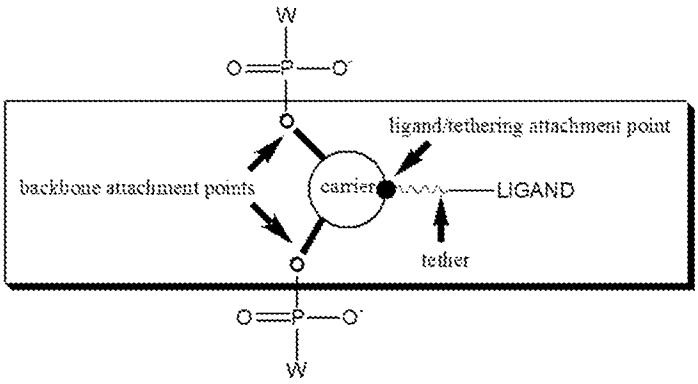
imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolane, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuryl, and decalin. In one embodiment, the acyclic group is a moiety based on a serinol backbone or a diethanolamine backbone.

[0383] The carrier can replace one or more nucleotide(s) of the iRNA agent.

[0384] In some embodiments, the carrier replaces one or more nucleotide(s) in the internal position(s) of the iRNA agent.

[0385] In other embodiments, the carrier replaces the nucleotides at the terminal end of the sense strand or antisense strand. In one embodiment, the carrier replaces the terminal nucleotide on the 3' end of the sense strand, thereby functioning as an end cap protecting the 3' end of the sense strand. In one embodiment, the carrier is a cyclic group having an amine, for instance, the carrier may be pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolanyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuranyl, or decalinyl.

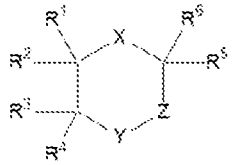
[0386] A ribonucleotide subunit in which the ribose sugar of the subunit has been so replaced is referred to herein as a ribose replacement modification subunit (RRMS). The carrier can be a cyclic or acyclic moiety and include two "backbone attachment points" (e.g., hydroxyl groups) and a ligand. The targeting ligand can be directly attached to the carrier or indirectly attached to the carrier by an intervening linker/tether, as described above.



[0387] The ligand-conjugated monomer subunit may be the 5' or 3' terminal subunit of the iRNA molecule, i.e., one of the two "W" groups may be a hydroxyl group, and the other "W" group may be a chain of two or more unmodified or modified ribonucleotides. Alternatively, the ligand-conjugated monomer subunit may occupy an internal position, and both "W" groups may be one or more unmodified or modified ribonucleotides. More than one ligand-conjugated monomer subunit may be present in an iRNA agent.

Sugar Replacement-Based Monomers, e.g., Ligand-Conjugated Monomers (Cyclic)

[0388] Cyclic sugar replacement-based monomers, e.g., sugar replacement-based ligand-conjugated monomers, are also referred to herein as RRMS monomer compounds. The carriers may have the general formula (**LCM-2**) provided below (In that structure preferred backbone attachment points can be chosen from R^1 or R^2; R^3 or R^4; or R^9 and R^{10} if Y is CR^9R^{10} (two positions are chosen to give two backbone attachment points, e.g., R^1 and R^4, or R^4 and R^9)). Preferred tethering attachment points include R^7; R^5 or R^6 when X is CH_2. The carriers are described below as an entity, which can be incorporated into a strand. Thus, it is understood that the structures also encompass the situations wherein one (in the case of a terminal position) or two (in the case of an internal position) of the attachment points, e.g., R^1 or R^2; R^3 or R^4; or R^9 or R^{10} (when Y is CR^9R^{10}), is connected to the phosphate, or modified phosphate, e.g., sulfur containing, backbone. E.g., one of the above-named R groups can be -CH_2-, wherein one bond is connected to the carrier and one to a backbone atom, e.g., a linking oxygen or a central phosphorus atom.



(LCM-2)

wherein:

X is $N(CO)R^7$, NR^7 or CH_2;

Y is NR^8, O, S, CR^9R^{10};

Z is $CR^{11}R^{12}$ or absent;

Each of R^1, R^2, R^3, R^4, R^9, and R^{10} is, independently, H, OR^a, or $(CH_2)_nOR^b$, provided that at least two of R^1, R^2, R^3, R^4, R^9, and R^{10} are OR^a and/or $(CH_2)_nOR^b$;

Each of R^5, R^6, R^{11}, and R^{12} is, independently, a ligand, H, C_1-C_6 alkyl optionally substituted with 1-3 R^{13}, or $C(O)NHR^7$; or R^5 and R^{11} together are C_3-C_8 cycloalkyl optionally substituted with R^{14};

R^7 can be a ligand, e.g., R^7 can be R^d , or R^7 can be a ligand tethered indirectly to the carrier, e.g., through a tethering moiety, e.g., C_1-C_{20} alkyl substituted with NR^cR^d; or C_1-C_{20} alkyl substituted with $NHC(O)R^d$;

R^8 is H or C_1-C_6 alkyl;

R^{13} is hydroxy, C_1-C_4 alkoxy, or halo;

R^{14} is NR^cR^7;

R^{15} is C_1-C_6 alkyl optionally substituted with cyano, or C_2-C_6 alkenyl;

R^{16} is C_1-C_{10} alkyl;

R^{17} is a liquid or solid phase support reagent;

L is $-C(O)(CH_2)_qC(O)-$, or $-C(O)(CH_2)_qS-$;

R^a is a protecting group, e.g., CAr_3; (e.g., a dimethoxytrityl group) or $Si(X^{5'})(X^{5''})(X^{5'''})$ in which $(X^{5'})$,$(X^{5''})$, and $(X^{5'''})$ are as described elsewhere.

R^b is $P(O)(O^-)H$, $P(OR^{15})N(R^{16})_2$ or $L\text{-}R^{17}$;

R^c is H or C_1-C_6 alkyl;

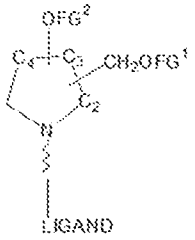
R^d is H or a ligand;

Each Ar is, independently, C_6-C_{10} aryl optionally substituted with C_1-C_4 alkoxy;

n is 1-4; and q is 0-4.

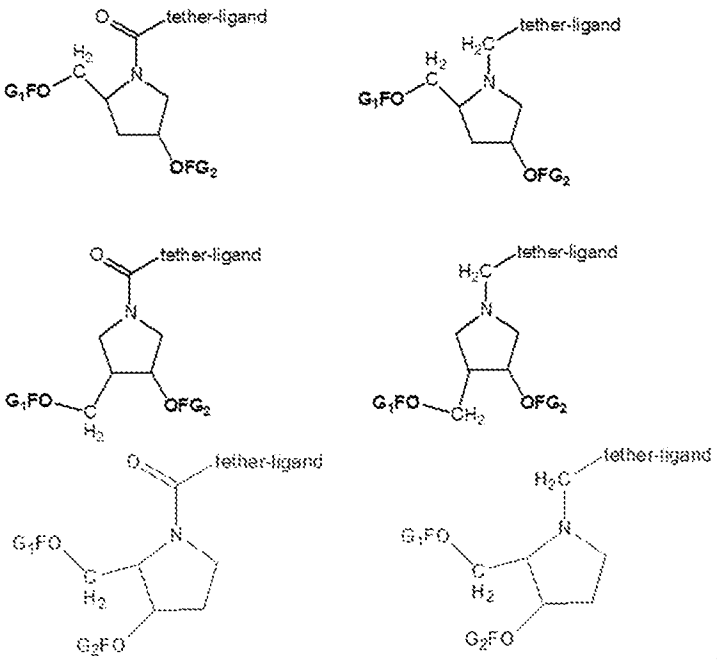
[0389] Exemplary carriers include those in which, *e.g.*, X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is absent; or X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is $CR^{11}R^{12}$; or X is $N(CO)R^7$ or NR^7, Y is NR^8, and Z is $CR^{11}R^{12}$; or X is $N(CO)R^7$ or NR^7, Y is O, and Z is $CR^{11}R^{12}$; or X is CH_2; Y is CR^9R^{10}; Z is $CR^{11}R^{12}$, and R^5 and R^{11} together form C_6 cycloalkyl (**H**, z = 2), or the indane ring system, *e.g.*, X is CH_2; Y is CR^9R^{10}; Z is $CR^{11}R^{12}$, and R^5 and R^{11} together form C_5 cycloalkyl (**H**, z = 1).

[0390] In certain embodiments, the carrier may be based on the pyrroline ring system or the 4-hydroxyproline ring system, *e.g.*, X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is absent

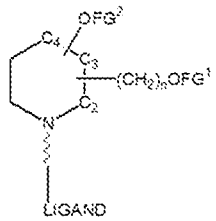


(**D**). . OFG^1 is preferably attached to a primary carbon, *e.g.*, an exocyclic alkylene group, *e.g.*, a methylene group, connected to one of the carbons in the five-membered ring ($-CH_2OFG^1$ in **D**). OFG^2 is preferably attached directly to one of the carbons in the five-membered ring ($-OFG^2$ in **D**). For the pyrroline-based carriers, $-CH_2OFG^1$ may be attached to C-2 and OFG^2 may be attached to C-3; or $-CH_2OFG^1$ may be attached to C-3 and OFG^2 may be attached to C-4. In certain embodiments, CH_2OFG^1 and OFG^2 may be geminally substituted to one of the above-referenced carbons. For the 3-hydroxyproline-based carriers, $-CH_2OFG^1$ may be attached to C-2 and OFG^2 may be attached to C-4. The pyrroline- and 4-hydroxyproline-based monomers may therefore contain linkages (*e.g.*,

carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, *e.g.* restriction resulting from the presence of a ring. Thus, CH_2OFG^1 and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included (e.g., the centers bearing CH_2OFG^1 and OFG^2 can both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and *vice versa*). The tethering attachment point is preferably nitrogen. Preferred examples of carrier **D** include the following:



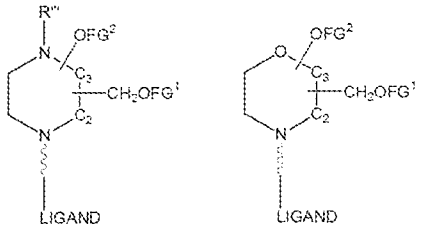
[0391] In certain embodiments, the carrier may be based on the piperidine ring system



(**E**), e.g., X is $N(CO)R^7$ or NR^7, Y is CR^9R^{10}, and Z is $CR^{11}R^{12}$. E . OFG^1 is preferably attached to a primary carbon, e.g., an exocyclic alkylene group, e.g., a methylene group (n=1) or ethylene group (n=2), connected to one of the carbons in the six-membered ring [$-(CH_2)_nOFG^1$ in **E**]. OFG^2 is preferably attached directly to one of the carbons in the six-membered ring ($-OFG^2$ in **E**). $-(CH_2)_nOFG^1$ and OFG^2 may be disposed in

a geminal manner on the ring, i.e., both groups may be attached to the same carbon, e.g., at C-2, C-3, or C-4. Alternatively, $-(CH_2)_nOFG^1$ and OFG^2 may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g., $-(CH_2)_nOFG^1$ may be attached to C-2 and OFG^2 may be attached to C-3; $-(CH_2)_nOFG^1$ may be attached to C-3 and OFG^2 may be attached to C-2; $-(CH_2)_nOFG^1$ may be attached to C-3 and OFG^2 may be attached to C-4; or $-(CH_2)_nOFG^1$ may be attached to C-4 and OFG^2 may be attached to C-3. The piperidine-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, $-(CH_2)_nOFG^1$ and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included (e.g., the centers bearing CH_2OFG^1 and OFG^2 can both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and *vice versa*). The tethering attachment point is preferably nitrogen.

[0392] In certain embodiments, the carrier may be based on the piperazine ring system (**F**), e.g., X is $N(CO)R^7$ or NR^7, Y is NR^8, and Z is $CR^{11}R^{12}$, or the morpholine ring system (**G**), e.g., X is $N(CO)R^7$ or NR^7, Y is O, and Z is $CR^{11}R^{12}$.

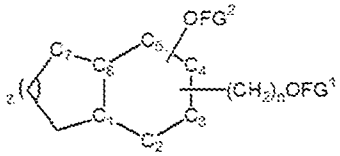


F G

OFG^1 is preferably attached to a primary carbon, e.g., an exocyclic alkylene group, e.g., a methylene group, connected to one of the carbons in the six-membered ring ($-CH_2OFG^1$ in **F** or **G**). OFG^2 is preferably attached directly to one of the carbons in the six-membered rings ($-OFG^2$ in **F** or **G**). For both **F** and **G**, $-CH_2OFG^1$ may be attached to C-2 and OFG^2 may be attached to C-3; or *vice versa*. In certain embodiments, CH_2OFG^1 and OFG^2 may be geminally substituted to one of the above-referenced carbons. The piperazine- and morpholine-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, CH_2OFG^1 and OFG^2 may be *cis* or *trans* with respect to one

another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included (e.g., the centers bearing CH_2OFG^1 and OFG^2 can both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and *vice versa*). R''' can be, e.g., C_1-C_6 alkyl, preferably CH_3. The tethering attachment point is preferably nitrogen in both **F** and **G**.

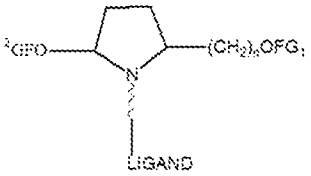
[0393] In certain embodiments, the carrier may be based on the decalin ring system, e.g., X is CH_2; Y is CR^9R^{10}; Z is $CR^{11}R^{12}$, and R^5 and R^{11} together form C_6 cycloalkyl (**H**, z = 2), or the indane ring system, e.g., X is CH_2; Y is CR^9R^{10}; Z is $CR^{11}R^{12}$, and R^5 and R^{11} together form C_5 cycloalkyl (**H**, z = 1).



. OFG^1 is preferably attached to a primary carbon, e.g., an exocyclic methylene group (n=1) or ethylene group (n=2) connected to one of C-2, C-3, C-4, or C-5 [$-(CH_2)_nOFG^1$ in **H**]. OFG^2 is preferably attached directly to one of C-2, C-3, C-4, or C-5 ($-OFG^2$ in **H**). $-(CH_2)_nOFG^1$ and OFG^2 may be disposed in a geminal manner on the ring, i.e., both groups may be attached to the same carbon, e.g., at C-2, C-3, C-4, or C-5. Alternatively, $-(CH_2)_nOFG^1$ and OFG^2 may be disposed in a vicinal manner on the ring, i.e., both groups may be attached to adjacent ring carbon atoms, e.g., $-(CH_2)_nOFG^1$ may be attached to C-2 and OFG^2 may be attached to C-3; $-(CH_2)_nOFG^1$ may be attached to C-3 and OFG^2 may be attached to C-2; $-(CH_2)_nOFG^1$ may be attached to C-3 and OFG^2 may be attached to C-4; or $-(CH_2)_nOFG^1$ may be attached to C-4 and OFG^2 may be attached to C-3; $-(CH_2)_nOFG^1$ may be attached to C-4 and OFG^2 may be attached to C-5; or $-(CH_2)_nOFG^1$ may be attached to C-5 and OFG^2 may be attached to C-4. The decalin or indane-based monomers may therefore contain linkages (e.g., carbon-carbon bonds) wherein bond rotation is restricted about that particular linkage, e.g. restriction resulting from the presence of a ring. Thus, $-(CH_2)_nOFG^1$ and OFG^2 may be *cis* or *trans* with respect to one another in any of the pairings delineated above. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly

included (e.g., the centers bearing CH_2OFG^1 and OFG^2 can both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and *vice versa*). In a preferred embodiment, the substituents at C-1 and C-6 are *trans* with respect to one another. The tethering attachment point is preferably C-6 or C-7.

[0394] Other carriers may include those based on 3-hydroxyproline (**J**).

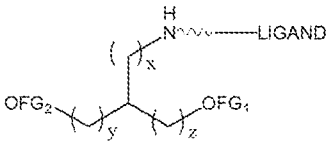


. Thus, $-(CH_2)_nOFG^1$ and OFG^2 may be *cis* or *trans* with respect to one another. Accordingly, all *cis/trans* isomers are expressly included. The monomers may also contain one or more asymmetric centers and thus occur as racemates and racemic mixtures, single enantiomers, individual diastereomers and diastereomeric mixtures. All such isomeric forms of the monomers are expressly included (e.g., the centers bearing CH_2OFG^1 and OFG^2 can both have the R configuration; or both have the S configuration; or one center can have the R configuration and the other center can have the S configuration and *vice versa*). The tethering attachment point is preferably nitrogen.

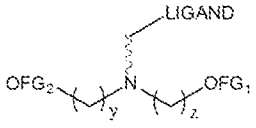
[0395] Details about more representative cyclic, sugar replacement-based carriers can be found in U.S. Patent Nos. 7,745,608 and 8,017,762, which are herein incorporated by reference in their entireties.

Sugar Replacement-Based Monomers (Acyclic)

[0396] Acyclic sugar replacement-based monomers, e.g., sugar replacement-based ligand-conjugated monomers, are also referred to herein as ribose replacement monomer subunit (RRMS) monomer compounds. Preferred acyclic carriers can have formula **LCM-3** or **LCM-4**:



LCM-3



LCM-4

[0397] In some embodiments, each of x, y, and z can be, independently of one another, 0, 1, 2, or 3. In formula **LCM-3**, when y and z are different, then the tertiary carbon can have either the R or S configuration. In preferred embodiments, x is zero and y and z are each 1 in

formula **LCM-3** (e.g., based on serinol), and y and z are each 1 in formula **LCM-3**. Each of formula **LCM-3** or **LCM-4** below can optionally be substituted, e.g., with hydroxy, alkoxy, perhaloalkyl.

[0398] Details about more representative acyclic, sugar replacement-based carriers can be found in U.S. Patent Nos. 7,745,608 and 8,017,762, which are herein incorporated by reference in their entireties.

[0399] In some embodiments, the oligonucleotide comprises one or more targeting ligands conjugated to the 5′ end of the sense strand or the 5’ end of the antisense strand, optionally via a carrier and/or linker/tether.

[0400] In some embodiments, the oligonucleotide comprises one or more targeting ligands conjugated to the 3′ end of the sense strand or the 3’ end of the antisense strand, optionally via a carrier and/or linker/tether.

[0401] In some embodiments, the oligonucleotide comprises one or more targeting ligands conjugated to both ends of the sense strand, optionally via a carrier and/or linker/tether.

[0402] In some embodiments, the oligonucleotide comprises one or more more targeting ligands conjugated to both ends of the antisense strand, optionally via a carrier and/or linker/tether.

[0403] In some embodiments, the oligonucleotide comprises one or more more targeting ligands conjugated to internal position(s) of the sense or antisense strand, optionally via a carrier and/or linker/tether.

[0404] In some embodiments, one or more targeting ligands are conjugated to the ribose, nucleobase, and/or at the internucleotide linkages. In some embodiments, one or more targeting ligands are conjugated to the ribose at the 2’ position, 3’ position, 4’ position, and/or 5’ position of the ribose. In some embodiments, one or more targeting ligands are conjugated at the nucleobase of natural (such as A, T, G, C, or U) or modified as defined herein. In some embodiments, one or more targeting ligands are conjugated at the phosphate or modified phosphate groups as defined herein.

[0405] In some embodiments, the oligonucleotide comprises one or more targeting ligands conjugated to the 5′ end or 3′ end of the sense strand, and one or more same or different targeting ligands conjugated to the 5′ end or 3′ end of the antisense strand,

[0406] In some embodiments, at least one targeting ligand is located on one or more terminal positions of the sense strand or antisense strand. In one embodiment, at least one

targeting ligand is located on the 3' end or 5' end of the sense strand. In one embodiment, at least one targeting ligand is located on the 3' end or 5' end of the antisense strand.

[0407] In some embodiments, at least one targeting ligand is conjugated to one or more internal positions on at least one strand. Internal positions of a strand refers to the nucleotide on any position of the strand, except the terminal position from the 3' end and 5' end of the strand (e.g., excluding 2 positions: position 1 counting from the 3' end and position 1 counting from the 5' end).

[0408] In one embodiment, at least one targeting ligand is located on one or more internal positions on at least one strand, which include all positions except the terminal two positions from each end of the strand (e.g., excluding 4 positions: positions 1 and 2 counting from the 3' end and positions 1 and 2 counting from the 5' end). In one embodiment, the targeting ligand is located on one or more internal positions on at least one strand, which include all positions except the terminal three positions from each end of the strand (e.g., excluding 6 positions: positions 1, 2, and 3 counting from the 3' end and positions 1, 2, and 3 counting from the 5' end).

[0409] In one embodiment, at least one targeting ligand is located on one or more positions of at least one end of the duplex region, which include all positions within the duplex region, but not include the overhang region or the carrier that replaces the terminal nucleotide on the 3' end of the sense strand.

[0410] In one embodiment, at least one targeting ligand is located on the sense strand within the first five, four, three, two, or first base pairs at the 5'-end of the antisense strand of the duplex region.

[0411] In one embodiment, at least one targeting ligand (e.g., a lipophilic moiety) is located on one or more internal positions on at least one strand, except the cleavage site region of the sense strand, for instance, the targeting ligand (e.g., a lipophilic moiety) is not located on positions 9-12 counting from the 5'-end of the sense strand, for example, the targeting ligand (e.g., a lipophilic moiety) is not located on positions 9-11 counting from the 5'-end of the sense strand. Alternatively, the internal positions exclude positions 11-13 counting from the 3'-end of the sense strand.

[0412] In one embodiment, at least one targeting ligand (e.g., a lipophilic moiety) is located on one or more internal positions on at least one strand, which exclude the cleavage site region of the antisense strand. For instance, the internal positions exclude positions 12-14 counting from the 5'-end of the antisense strand.

[0413] In one embodiment, at least one targeting ligand (e.g., a lipophilic moiety) is located on one or more internal positions on at least one strand, which exclude positions 11-13 on the sense strand, counting from the 3'-end, and positions 12-14 on the antisense strand, counting from the 5'-end.

[0414] In one embodiment, one or more targeting ligands (e.g., a lipophilic moiety) are located on one or more of the following internal positions: positions 4-8 and 13-18 on the sense strand, and positions 6-10 and 15-18 on the antisense strand, counting from the 5'end of each strand.

[0415] In one embodiment, one or more targeting ligands (e.g., a lipophilic moiety) are located on one or more of the following internal positions: positions 5, 6, 7, 15, and 17 on the sense strand, and positions 15 and 17 on the antisense strand, counting from the 5'end of each strand.

Target genes

[0416] Without limitations, target genes for siRNAs include, but are not limited to genes promoting unwanted cell proliferation, growth factor gene, growth factor receptor gene, genes expressing kinases, an adaptor protein gene, a gene encoding a G protein super family molecule, a gene encoding a transcription factor, a gene which mediates angiogenesis, a viral gene, a gene required for viral replication, a cellular gene which mediates viral function, a gene of a bacterial pathogen, a gene of an amoebic pathogen, a gene of a parasitic pathogen, a gene of a fungal pathogen, a gene which mediates an unwanted immune response, a gene which mediates the processing of pain, a gene which mediates a neurological disease, an allene gene found in cells characterized by loss of heterozygosity, or one allege gene of a polymorphic gene.

[0417] Specific exemplary target genes for the siRNAs include, but are not limited to, PCSK-9, ApoC3, AT3, AGT, ALAS1, TMPR, HAO1, AGT, C5, CCR-5, PDGF beta gene; Erb-B gene, Src gene; CRK gene; GRB2 gene; RAS gene; MEKK gene; JNK gene; RAF gene; Erk1/2 gene; PCNA(p21) gene; MYB gene; c-MYC gene; JUN gene; FOS gene; BCL-2 gene; Cyclin D gene; VEGF gene; EGFR gene; Cyclin A gene; Cyclin E gene; WNT-1 gene; beta-catenin gene; c-MET gene; PKC gene; NFKB gene; STAT3 gene; survivin gene; Her2/Neu gene; topoisomerase I gene; topoisomerase II alpha gene; p73 gene; p21(WAF1/CIP1) gene, p27(KIP1) gene; PPM1D gene; caveolin I gene; MIB I gene; MTAI gene; M68 gene; tumor suppressor genes; p53 gene; DN-p63 gene; pRb tumor suppressor

gene; APC1 tumor suppressor gene; BRCA1 tumor suppressor gene; PTEN tumor suppressor gene; MLL fusion genes, e.g., MLL-AF9, BCR/ABL fusion gene; TEL/AML1 fusion gene; EWS/FLI1 fusion gene; TLS/FUS1 fusion gene; PAX3/FKHR fusion gene; AML1/ETO fusion gene; alpha v-integrin gene; Flt-1 receptor gene; tubulin gene; Human Papilloma Virus gene, a gene required for Human Papilloma Virus replication, Human Immunodeficiency Virus gene, a gene required for Human Immunodeficiency Virus replication, Hepatitis A Virus gene, a gene required for Hepatitis A Virus replication, Hepatitis B Virus gene, a gene required for Hepatitis B Virus replication, Hepatitis C Virus gene, a gene required for Hepatitis C Virus replication, Hepatitis D Virus gene, a gene required for Hepatitis D Virus replication, Hepatitis E Virus gene, a gene required for Hepatitis E Virus replication, Hepatitis F Virus gene, a gene required for Hepatitis F Virus replication, Hepatitis G Virus gene, a gene required for Hepatitis G Virus replication, Hepatitis H Virus gene, a gene required for Hepatitis H Virus replication, Respiratory Syncytial Virus gene, a gene that is required for Respiratory Syncytial Virus replication, Herpes Simplex Virus gene, a gene that is required for Herpes Simplex Virus replication, herpes Cytomegalovirus gene, a gene that is required for herpes Cytomegalovirus replication, herpes Epstein Barr Virus gene, a gene that is required for herpes Epstein Barr Virus replication, Kaposi's Sarcoma-associated Herpes Virus gene, a gene that is required for Kaposi's Sarcoma-associated Herpes Virus replication, JC Virus gene, human gene that is required for JC Virus replication, myxovirus gene, a gene that is required for myxovirus gene replication, rhinovirus gene, a gene that is required for rhinovirus replication, coronavirus gene, a gene that is required for coronavirus replication, West Nile Virus gene, a gene that is required for West Nile Virus replication, St. Louis Encephalitis gene, a gene that is required for St. Louis Encephalitis replication, Tick-borne encephalitis virus gene, a gene that is required for Tick-borne encephalitis virus replication, Murray Valley encephalitis virus gene, a gene that is required for Murray Valley encephalitis virus replication, dengue virus gene, a gene that is required for dengue virus gene replication, Simian Virus 40 gene, a gene that is required for Simian Virus 40 replication, Human T Cell Lymphotropic Virus gene, a gene that is required for Human T Cell Lymphotropic Virus replication, Moloney-Murine Leukemia Virus gene, a gene that is required for Moloney-Murine Leukemia Virus replication, encephalomyocarditis virus gene, a gene that is required for encephalomyocarditis virus replication, measles virus gene, a gene that is required for measles virus replication, Vericella zoster virus gene, a gene that is required for Vericella zoster virus replication, adenovirus gene, a gene that is required for adenovirus replication,

yellow fever virus gene, a gene that is required for yellow fever virus replication, poliovirus gene, a gene that is required for poliovirus replication, poxvirus gene, a gene that is required for poxvirus replication, plasmodium gene, a gene that is required for plasmodium gene replication, Mycobacterium ulcerans gene, a gene that is required for Mycobacterium ulcerans replication, Mycobacterium tuberculosis gene, a gene that is required for Mycobacterium tuberculosis replication, Mycobacterium leprae gene, a gene that is required for Mycobacterium leprae replication, Staphylococcus aureus gene, a gene that is required for Staphylococcus aureus replication, Streptococcus pneumoniae gene, a gene that is required for Streptococcus pneumoniae replication, Streptococcus pyogenes gene, a gene that is required for Streptococcus pyogenes replication, Chlamydia pneumoniae gene, a gene that is required for Chlamydia pneumoniae replication, Mycoplasma pneumoniae gene, a gene that is required for Mycoplasma pneumoniae replication, an integrin gene, a selectin gene, complement system gene, chemokine gene, chemokine receptor gene, GCSF gene, Gro1 gene, Gro2 gene, Gro3 gene, PF4 gene, MIG gene, Pro-Platelet Basic Protein gene, MIP-1I gene, MIP-1J gene, RANTES gene, MCP-1 gene, MCP-2 gene, MCP-3 gene, CMBKR1 gene, CMBKR2 gene, CMBKR3 gene, CMBKR5v, AIF-1 gene, I-309 gene, a gene to a component of an ion channel, a gene to a neurotransmitter receptor, a gene to a neurotransmitter ligand, amyloid-family gene, presenilin gene, HD gene, DRPLA gene, SCA1 gene, SCA2 gene, MJD1 gene, CACNL1A4 gene, SCA7 gene, SCA8 gene, allele gene found in loss of heterozygosity (LOH) cells, one allele gene of a polymorphic gene and combinations thereof.

[0418] The loss of heterozygosity (LOH) can result in hemizygosity for sequence, e.g., genes, in the area of LOH. This can result in a significant genetic difference between normal and disease-state cells, e.g., cancer cells, and provides a useful difference between normal and disease-state cells, e.g., cancer cells. This difference can arise because a gene or other sequence is heterozygous in duploid cells but is hemizygous in cells having LOH. The regions of LOH will often include a gene, the loss of which promotes unwanted proliferation, e.g., a tumor suppressor gene, and other sequences including, e.g., other genes, in some cases a gene which is essential for normal function, e.g., growth. Methods of the invention rely, in part, on the specific modulation of one allele of an essential gene with a composition of the invention.

[0419] In certain embodiments, the invention provides an olignucleotide that modulates a micro-RNA.

Targeting CNS

[0420] In some embodiments, the invention provides an oligonucleotide that targets APP for Early Onset Familial Alzheimer Disease, ATXN2 for Spinocerebellar Ataxia 2 and ALS, and C9orf72 for Amyotrophic Lateral Sclerosis and Frontotemporal Dementia.

[0421] In some embodiments, the invention provides an oligonucleotide that targets TARDBP for ALS, MAPT (Tau) for Frontotemporal Dementia, and HTT for Huntington Disease.

[0422] In some embodiments, the invention provides an oligonucleotide that targets SNCA for Parkinson Disease, FUS for ALS, ATXN3 for Spinocerebellar Ataxia 3, ATXN1 for SCA1, genes for SCA7 and SCA8, ATN1 for DRPLA, MeCP2 for XLMR, PRNP for Prion Diseases, recessive CNS disorders: Lafora Disease, DMPK for DM1 (CNS and Skeletal Muscle), and TTR for hATTR (CNS, ocular and systemic).

[0423] Spinocerebellar ataxia is an inherited brain-function disorder. Dominantly inherited forms of spinocerebellar ataxias, such as SCA1-8, are devastating disorders with no disease-modifying therapy. Exemplary targets include SCA2, SCA3, and SCA1.

[0424] More detailed descriptions about these CNS targeting receptors and related diseases may be found in PCT Application No. PCT/US20/59399, entitled "Extrahepatic Delivery," filed on November 6, 2020, the content of which is incorporated herein by reference in its entirety.

[0425] In some embodiments, the invention provides an oligonucleotide that target genes for diseases including, but are not limited to, age-related macular degeneration (AMD) (dry and wet), birdshot chorioretinopathy, dominant retinitis pigmentosa 4, Fuch's dystrophy, hATTR amyloidosis, hereditary and sporadic glaucoma, and stargardt's disease.

[0426] In some embodiments, the oligonucleotide targets VEGF for wet (or exudative) AMD.

[0427] In some embodiments, the oligonucleotide targets C3 for dry (or nonexudative) AMD.

[0428] In some embodiments, the oligonucleotide targets CFB for dry (or nonexudative) AMD.

[0429] In some embodiments, the oligonucleotide targets MYOC for glaucoma.

[0430] In some embodiments, the oligonucleotide targets ROCK2 for glaucoma.

[0431] In some embodiments, the oligonucleotide targets ADRB2 for glaucoma.

[0432] In some embodiments, the oligonucleotide targets CA2 for glaucoma.

[0433] In some embodiments, the oligonucleotide targets CRYGC for cataract.

[0434] In some embodiments, the oligonucleotide targets PPP3CB for dry eye syndrome.

Ligands

[0435] In certain embodiments, the oligonucleotide is further modified by covalent attachment of one or more conjugate groups. In general, conjugate groups modify one or more properties of the attached compound of the invention including but not limited to pharmacodynamic, pharmacokinetic, binding, absorption, cellular distribution, cellular uptake, charge and clearance. Conjugate groups are routinely used in the chemical arts and are linked directly or via an optional linking moiety or linking group to a parent compound such as an oligonucleotide. A preferred list of conjugate groups includes without limitation, intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, thioethers, polyethers, cholesterols, thiocholesterols, cholic acid moieties, folate, lipids, phospholipids, biotin, phenazine, phenanthridine, anthraquinone, adamantane, acridine, fluoresceins, rhodamines, coumarins and dyes.

[0436] In some embodiments, the oligonucleotide further comprises a targeting ligand that targets a receptor which mediates delivery to a specific CNS tissue. These targeting ligands can also be conjugated in combination with a lipophilic moiety to enable specific intrathecal and systemic delivery.

[0437] Exemplary targeting ligands that targets the receptor mediated delivery to a CNS tissue are peptide ligands such as Angiopep-2, lipoprotein receptor related protein (LRP) ligand, bEnd.3 cell binding ligand; transferrin receptor (TfR) ligand (which can utilize iron transport system in brain and cargo transport into the brain parenchyma); manose receptor ligand (which targets olfactory ensheathing cells, glial cells), glucose transporter protein, and LDL receptor ligand.

[0438] In some embodiments, the oligonucleotide further comprises a targeting ligand that targets a receptor which mediates delivery to a specific ocular tissue. These targeting ligands can also be conjugated in combination with a lipophilic moiety to enable specific ocular delivery (e.g., intravitreal delivery) and systemic delivery. Exemplary targeting ligands that targets the receptor mediated delivery to a ocular tissue are lipophilic ligands such as all-trans retinol (which targets the retinoic acid receptor); RGD peptide (which targets retinal pigment epithelial cells), such as H-Gly-Arg-Gly-Asp-Ser-Pro-Lys-Cys-OH or Cyclo(-Arg-Gly-Asp-D-Phe-Cys; LDL receptor ligands; and carbohydrate based ligands

(which targets endothelial cells in posterior eye).

[0439] Preferred conjugate groups amenable to the present invention include lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553); cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053); a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765); a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533); an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 111; Kabanov et al., FEBS Lett., 1990, 259, 327; Svinarchuk et al., Biochimie, 1993, 75, 49); a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium-1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651; Shea et al., Nucl. Acids Res., 1990, 18, 3777); a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969); adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651); a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229); or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923).

[0440] Generally, a wide variety of entities, e.g., ligands, can be coupled to the oligonucleotides described herein. Ligands can include naturally occurring molecules, or recombinant or synthetic molecules. Exemplary ligands include, but are not limited to, polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG, *e.g.*, PEG-2K, PEG-5K, PEG-10K, PEG-12K, PEG-15K, PEG-20K, PEG-40K), MPEG, $[MPEG]_2$, polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, polyphosphazine, polyethylenimine, cationic groups, spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, mucin, glycosylated polyaminoacids, transferrin, bisphosphonate, polyglutamate, polyaspartate, aptamer, asialofetuin, hyaluronan, procollagen, immunoglobulins (e.g., antibodies), insulin, transferrin, albumin, sugar-albumin conjugates, intercalating agents (*e.g.*, acridines), cross-linkers (*e.g.* psoralen, mitomycin C), porphyrins (e.g., TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial

endonucleases (*e.g.*, EDTA), lipophilic molecules (e.g., steroids, bile acids, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine), peptides (e.g., an alpha helical peptide, amphipathic peptide, RGD peptide, cell permeation peptide, endosomolytic/fusogenic peptide), alkylating agents, phosphate, amino, mercapto, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, naproxen, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, AP, antibodies, hormones and hormone receptors, lectins, carbohydrates, multivalent carbohydrates, vitamins (e.g., vitamin A, vitamin E, vitamin K, vitamin B, e.g., folic acid, B12, riboflavin, biotin and pyridoxal), vitamin cofactors, lipopolysaccharide, an activator of p38 MAP kinase, an activator of NF-κB, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, myoservin, tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, gamma interferon, natural or recombinant low density lipoprotein (LDL), natural or recombinant high-density lipoprotein (HDL), and a cell-permeation agent (e.g., a helical cell-permeation agent).

[0441] Peptide and peptidomimetic ligands include those having naturally occurring or modified peptides, e.g., D or L peptides; α, β, or γ peptides; N-methyl peptides; azapeptides; peptides having one or more amide, i.e., peptide, linkages replaced with one or more urea, thiourea, carbamate, or sulfonyl urea linkages; or cyclic peptides. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The peptide or peptidomimetic ligand can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[0442] Exemplary amphipathic peptides include, but are not limited to, cecropins, lycotoxins, paradaxins, buforin, CPF, bombinin-like peptide (BLP), cathelicidins, ceratotoxins, *S. clava* peptides, hagfish intestinal antimicrobial peptides (HFIAPs), magainines, brevinins-2, dermaseptins, melittins, pleurocidin, H_2A peptides, Xenopus peptides, esculentinis-1, and caerins.

[0443] As used herein, the term "endosomolytic ligand" refers to molecules having

endosomolytic properties. Endosomolytic ligands promote the lysis of and/or transport of the composition of the invention, or its components, from the cellular compartments such as the endosome, lysosome, endoplasmic reticulum (ER), Golgi apparatus, microtubule, peroxisome, or other vesicular bodies within the cell, to the cytoplasm of the cell. Some exemplary endosomolytic ligands include, but are not limited to, imidazoles, poly or oligoimidazoles, linear or branched polyethyleneimines (PEIs), linear and branched polyamines, e.g. spermine, cationic linear and branched polyamines, polycarboxylates, polycations, masked oligo or poly cations or anions, acetals, polyacetals, ketals/polyketals, orthoesters, linear or branched polymers with masked or unmasked cationic or anionic charges, dendrimers with masked or unmasked cationic or anionic charges, polyanionic peptides, polyanionic peptidomimetics, pH-sensitive peptides, natural and synthetic fusogenic lipids, natural and synthetic cationic lipids.

[0444] Exemplary endosomolytic/fusogenic peptides include, but are not limited to, AALEALAEALEALAEALEALAEAAAAGGC (GALA); AALAEALAEALAEALAEALAEALAAAAGGC (EALA); ALEALAEALEALAEA; GLFEAIEGFIENGWEGMIWDYG (INF-7); GLFGAIAGFIENGWEGMIDGWYG (Inf HA-2); GLFEAIEGFIENGWEGMIDGWYGCGLFEAIEGFIENGWEGMID GWYGC (diINF-7); GLFEAIEGFIENGWEGMIDGGCGLFEAIEGFIENGWEGMIDGGC (diINF-3); GLFGALAEALAEALAEHLAEALAEALEALAAGGSC (GLF); GLFEAIEGFIENGWEGLAEALAEALEALAAGGSC (GALA-INF3); GLF EAI EGFI ENGW EGnI DG K GLF EAI EGFI ENGW EGnI DG (INF-5, n is norleucine); LFEALLELLESLWELLLEA (JTS-1); GLFKALLKLLKSLWKLLLKA (ppTG1); GLFRALLRLLRSLWRLLLRA (ppTG20); WEAKLAKALAKALAKHLAKALAKALKACEA (KALA); GLFFEAIAEFIEGGWEGLIEGC (HA); GIGAVLKVLTTGLPALISWIKRKRQQ (Melittin); H_5WYG; and CHK_6HC.

[03100] Without wishing to be bound by theory, fusogenic lipids fuse with and consequently destabilize a membrane. Fusogenic lipids usually have small head groups and unsaturated acyl chains. Exemplary fusogenic lipids include, but are not limited to, 1,2-dileoyl-sn-3-phosphoethanolamine (DOPE), phosphatidylethanolamine (POPE), palmitoyloleoylphosphatidylcholine (POPC), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-ol (Di-Lin), N-methyl(2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-1,3-dioxolan-4-yl)methanamine (DLin-k-DMA) and N-methyl-2-(2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-

1,3-dioxolan-4-yl)ethanamine (also referred to as XTC herein).

[0445] Synthetic polymers with endosomolytic activity amenable to the present invention are described in U.S. Pat. App. Pub. Nos. 2009/0048410; 2009/0023890; 2008/0287630; 2008/0287628; 2008/0281044; 2008/0281041; 2008/0269450; 2007/0105804; 20070036865; and 2004/0198687, contents of which are hereby incorporated by reference in their entirety.

[0446] Exemplary cell permeation peptides include, but are not limited to, RQIKIWFQNRRMKWKK (penetratin); GRKKRRQRRRPPQC (Tat fragment 48-60); GALFLGWLGAAGSTMGAWSQPKKKRKV (signal sequence based peptide); LLIILRRRIRKQAHAHSK (PVEC); GWTLNSAGYLLKINLKALAALAKKIL (transportan); KLALKLALKALKAALKLA (amphiphilic model peptide); RRRRRRRRR (Arg9); KFFKFFKFFK (Bacterial cell wall permeating peptide); LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES (LL-37); SWLSKTAKKLENSAKKRISEGIAIAIQGGPR (cecropin P1); ACYCRIPACIAGERRYGTCIYQGRLWAFCC (α-defensin); DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK (β-defensin); RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFPGKR-NH2 (PR-39); ILPWKWPWWPWRR-NH2 (indolicidin); AAVALLPAVLLALLAP (RFGF); AALLPVLLAAP (RFGF analogue); and RKCRIVVIRVCR (bactenecin).

[0447] Exemplary cationic groups include, but are not limited to, protonated amino groups, derived from e.g., O-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); aminoalkoxy, e.g., $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); amino (*e.g.* NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); and $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino).

[0448] As used herein the term "targeting ligand" refers to any molecule that provides an enhanced affinity for a selected target, e.g., a cell, cell type, tissue, organ, region of the body, or a compartment, e.g., a cellular, tissue or organ compartment. Some exemplary targeting ligands include, but are not limited to, antibodies, antigens, folates, receptor ligands, carbohydrates, aptamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL and HDL

ligands.

[0449] Carbohydrate based targeting ligands include, but are not limited to, D-galactose, multivalent galactose, N-acetyl-D-galactosamine (GalNAc), multivalent GalNAc, e.g. $GalNAc_2$ and $GalNAc_3$ (GalNAc and multivalent GalNAc are collectively referred to herein as GalNAc conjugates); D-mannose, multivalent mannose, multivalent lactose, N-acetyl-glucosamine, Glucose, multivalent Glucose, multivalent fucose, glycosylated polyaminoacids and lectins. The term multivalent indicates that more than one monosaccharide unit is present. Such monosaccharide subunits can be linked to each other through glycosidic linkages or linked to a scaffold molecule.

[0450] A number of folate and folate analogs amenable to the present invention as ligands are described in U.S. Pat. Nos. 2,816,110; 5,552,545; 6,335,434 and 7,128,893, contents of which are herein incorporated in their entireties by reference.

[0451] As used herein, the terms "PK modulating ligand" and "PK modulator" refers to molecules which can modulate the pharmacokinetics of the composition of the invention. Some exemplary PK modulator include, but are not limited to, lipophilic molecules, bile acids, sterols, phospholipid analogues, peptides, protein binding agents, vitamins, fatty acids, phenoxazine, aspirin, naproxen, ibuprofen, suprofen, ketoprofen, (S)-(+)-pranoprofen, carprofen, PEGs, biotin, and transthyretia-binding ligands (e.g., tetraiidothyroacetic acid, 2, 4, 6-triiodophenol and flufenamic acid). Oligonucleotides that comprise a number of phosphorothioate intersugar linkages are also known to bind to serum protein, thus short oligonucleotides, e.g. oligonucleotides of comprising from about 5 to 30 nucleotides (e.g., 5 to 25 nucleotides, preferably 5 to 20 nucleotides, e.g., 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides), and that comprise a plurality of phosphorothioate linkages in the backbone are also amenable to the present invention as ligands (e.g. as PK modulating ligands). The PK modulating oligonucleotide can comprise at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more phosphorothioate and/or phosphorodithioate linkages. In some embodiments, all internucleotide linkages in PK modulating oligonucleotide are phosphorothioate and/or phosphorodithioates linkages. In addition, aptamers that bind serum components (e.g. serum proteins) are also amenable to the present invention as PK modulating ligands. Binding to serum components (e.g. serum proteins) can be predicted from albumin binding assays, such as those described in Oravcova, et al., Journal of Chromatography B (1996), 677: 1-27.

[0452] When two or more ligands are present, the ligands can all have same properties,

all have different properties or some ligands have the same properties while others have different properties. For example, a ligand can have targeting properties, have endosomolytic activity or have PK modulating properties. In a preferred embodiment, all the ligands have different properties.

[0453] The ligand or tethered ligand can be present on a monomer when said monomer is incorporated into a component of the compound of the invention (e.g., a compound of the invention or linker). In some embodiments, the ligand can be incorporated via coupling to a "precursor" monomer after said "precursor" monomer has been incorporated into a component of the compound of the invention (e.g., a compound of the invention or linker). For example, a monomer having, e.g., an amino-terminated tether (i.e., having no associated ligand), e.g., monomer-linker-NH_2 can be incorporated into a component of the compounds of the invention (e.g., a compound of the invention or linker). In a subsequent operation, i.e., after incorporation of the precursor monomer into a component of the compounds of the invention (e.g., a compound of the invention or linker), a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor monomer's tether.

[0454] In another example, a monomer having a chemical group suitable for taking part in Click Chemistry reaction can be incorporated e.g., an azide or alkyne terminated tether/linker. In a subsequent operation, i.e., after incorporation of the precursor monomer into the strand, a ligand having complementary chemical group, e.g. an alkyne or azide can be attached to the precursor monomer by coupling the alkyne and the azide together.

[0455] In some embodiments, ligand can be conjugated to nucleobases, sugar moieties, or internucleosidic linkages of the oligonucleotide. Conjugation to purine nucleobases or derivatives thereof can occur at any position including, endocyclic and exocyclic atoms. In some embodiments, the 2-, 6-, 7-, or 8-positions of a purine nucleobase are attached to a conjugate moiety. Conjugation to pyrimidine nucleobases or derivatives thereof can also occur at any position. In some embodiments, the 2-, 5-, and 6-positions of a pyrimidine nucleobase can be substituted with a conjugate moiety. When a ligand is conjugated to a nucleobase, the preferred position is one that does not interfere with hybridization, i.e., does not interfere with the hydrogen bonding interactions needed for base pairing.

[0456] Conjugation to sugar moieties of nucleosides can occur at any carbon atom. Exemplary carbon atoms of a sugar moiety that can be attached to a conjugate moiety include

the 2', 3', and 5' carbon atoms. The 1' position can also be attached to a conjugate moiety, such as in an abasic residue. Internucleosidic linkages can also bear conjugate moieties. For phosphorus-containing linkages (e.g., phosphodiester, phosphorothioate, phosphorodithioate, phosphoroamidate, and the like), the conjugate moiety can be attached directly to the phosphorus atom or to an O, N, or S atom bound to the phosphorus atom. For amine- or amide-containing internucleosidic linkages (e.g., PNA), the conjugate moiety can be attached to the nitrogen atom of the amine or amide or to an adjacent carbon atom.

[0457] There are numerous methods for preparing conjugates of oligonucleotides. Generally, an oligonucleotide is attached to a conjugate moiety by contacting a reactive group (e.g., OH, SH, amine, carboxyl, aldehyde, and the like) on the oligonucleotide with a reactive group on the conjugate moiety. In some embodiments, one reactive group is electrophilic and the other is nucleophilic.

[0458] For example, an electrophilic group can be a carbonyl-containing functionality and a nucleophilic group can be an amine or thiol. Methods for conjugation of nucleic acids and related oligonucleotides with and without linking groups are well described in the literature such as, for example, in Manoharan in Antisense Research and Applications, Crooke and LeBleu, eds., CRC Press, Boca Raton, Fla., 1993, Chapter 17, which is incorporated herein by reference in its entirety.

[0459] The ligand can be attached to the oligonucleotide via a linker or a carrier monomer, e.g., a ligand carrier. The carriers include (i) at least one "backbone attachment point," preferably two "backbone attachment points" and (ii) at least one "tethering attachment point." A "backbone attachment point" as used herein refers to a functional group, e.g. a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier monomer into the backbone, e.g., the phosphate, or modified phosphate, e.g., sulfur containing, backbone, of an oligonucleotide. A "tethering attachment point" (TAP) in refers to an atom of the carrier monomer, e.g., a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The selected moiety can be, e.g., a carbohydrate, e.g. monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide and polysaccharide. Optionally, the selected moiety is connected by an intervening tether to the carrier monomer. Thus, the carrier will often include a functional group, e.g., an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, e.g., a ligand to the constituent atom.

[0460] Representative U.S. patents that teach the preparation of conjugates of nucleic acids include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218, 105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578, 717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118, 802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578, 718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762, 779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904, 582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082, 830; 5,112,963; 5,149,782; 5,214,136; 5,245,022; 5,254, 469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317, 098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510, 475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574, 142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599, 923; 5,599,928; 5,672,662; 5,688,941; 5,714,166; 6,153, 737; 6,172,208; 6,300,319; 6,335,434; 6,335,437; 6,395, 437; 6,444,806; 6,486,308; 6,525,031; 6,528,631; 6,559, 279; contents of which are herein incorporated in their entireties by reference.

[0461] In some embodiments, the oligonucleotide further comprises a targeting ligand that targets a liver tissue. In some embodiments, the targeting ligand is a carbohydrate-based ligand. In one embodiment, the targeting ligand is a GalNAc conjugate.

[0462] Because the ligand can be conjugated to the iRNA agent via a linker or carrier, and because the linker or carrier can contain a branched linker, the iRNA agent can then contain multiple ligands via the same or different backbone attachment points to the carrier, or via the branched linker(s). For instance, the branchpoint of the branched linker may be a bivalent, trivalent, tetravalent, pentavalent, or hexavalent atom, or a group presenting such multiple valences. In certain embodiments, the branchpoint is -N, -N(Q)-C, -O-C, -S-C, -SS-C, -C(O)N(Q)-C, -OC(O)N(Q)-C, -N(Q)C(O)-C, or -N(Q)C(O)O-C; wherein Q is independently for each occurrence H or optionally substituted alkyl. In other embodiment, the branchpoint is glycerol or glycerol derivative.

Evaluation of Candidate iRNAs

[0463] One can evaluate a candidate iRNA agent, *e.g.*, a modified RNA, for a selected property by exposing the agent or modified molecule and a control molecule to the appropriate conditions and evaluating for the presence of the selected property. For example, resistance to a degradant can be evaluated as follows. A candidate modified RNA (and a control molecule, usually the unmodified form) can be exposed to degradative conditions, *e.g.*, exposed to a milieu, which includes a degradative agent, *e.g.*, a nuclease. *E.g.*, one can use a biological sample, *e.g.*, one that is similar to a milieu, which might be encountered, in

therapeutic use, *e.g.*, blood or a cellular fraction, *e.g.*, a cell-free homogenate or disrupted cells. The candidate and control could then be evaluated for resistance to degradation by any of a number of approaches. For example, the candidate and control could be labeled prior to exposure, with, *e.g.*, a radioactive or enzymatic label, or a fluorescent label, such as Cy3 or Cy5. Control and modified RNA's can be incubated with the degradative agent, and optionally a control, *e.g.*, an inactivated, *e.g.*, heat inactivated, degradative agent. A physical parameter, *e.g.*, size, of the modified and control molecules are then determined. They can be determined by a physical method, *e.g.*, by polyacrylamide gel electrophoresis or a sizing column, to assess whether the molecule has maintained its original length, or assessed functionally. Alternatively, Northern blot analysis can be used to assay the length of an unlabeled modified molecule.

[0464] A functional assay can also be used to evaluate the candidate agent. A functional assay can be applied initially or after an earlier non-functional assay, (*e.g.*, assay for resistance to degradation) to determine if the modification alters the ability of the molecule to silence gene expression. For example, a cell, *e.g.*, a mammalian cell, such as a mouse or human cell, can be co-transfected with a plasmid expressing a fluorescent protein, *e.g.*, GFP, and a candidate RNA agent homologous to the transcript encoding the fluorescent protein (see, *e.g.*, WO 00/44914). For example, a modified dsiRNA homologous to the GFP mRNA can be assayed for the ability to inhibit GFP expression by monitoring for a decrease in cell fluorescence, as compared to a control cell, in which the transfection did not include the candidate dsiRNA, *e.g.*, controls with no agent added and/or controls with a non-modified RNA added. Efficacy of the candidate agent on gene expression can be assessed by comparing cell fluorescence in the presence of the modified and unmodified dssiRNAs.

[0465] In an alternative functional assay, a candidate dssiRNA homologous to an endogenous mouse gene, for example, a maternally expressed gene, such as *c-mos*, can be injected into an immature mouse oocyte to assess the ability of the agent to inhibit gene expression *in vivo* (see, *e.g.*, WO 01/36646). A phenotype of the oocyte, *e.g.*, the ability to maintain arrest in metaphase II, can be monitored as an indicator that the agent is inhibiting expression. For example, cleavage of *c-mos* mRNA by a dssiRNA would cause the oocyte to exit metaphase arrest and initiate parthenogenetic development (Colledge *et al.* Nature 370: 65-68, 1994; Hashimoto *et al.* Nature, 370:68-71, 1994). The effect of the modified agent on target RNA levels can be verified by Northern blot to assay for a decrease in the level of target mRNA, or by Western blot to assay for a decrease in the level of target protein, as

compared to a negative control. Controls can include cells in which with no agent is added and/or cells in which a non-modified RNA is added.

Physiological Effects

[0466] The siRNAs described herein can be designed such that determining therapeutic toxicity is made easier by the complementarity of the siRNA with both a human and a non-human animal sequence. By these methods, an siRNA can consist of a sequence that is fully complementary to a nucleic acid sequence from a human *and* a nucleic acid sequence from at least one non-human animal, *e.g.*, a non-human mammal, such as a rodent, ruminant or primate. For example, the non-human mammal can be a mouse, rat, dog, pig, goat, sheep, cow, monkey, Pan paniscus, Pan troglodytes, Macaca mulatto, or Cynomolgus monkey. The sequence of the siRNA could be complementary to sequences within homologous genes, *e.g.*, oncogenes or tumor suppressor genes, of the non-human mammal and the human. By determining the toxicity of the siRNA in the non-human mammal, one can extrapolate the toxicity of the siRNA in a human. For a more strenuous toxicity test, the siRNA can be complementary to a human and more than one, *e.g.*, two or three or more, non-human animals.

[0467] The methods described herein can be used to correlate any physiological effect of an siRNA on a human, *e.g.*, any unwanted effect, such as a toxic effect, or any positive, or desired effect.

Increasing Cellular Uptake of siRNAs

[0468] Described herein are various siRNA compositions that contain covalently attached conjugates that increase cellular uptake and/or intracellular targeting of the siRNAs.

[0469] Additionally provided are methods of the invention that include administering an siRNA and a drug that affects the uptake of the siRNA into the cell. The drug can be administered before, after, or at the same time that the siRNA is administered. The drug can be covalently or non-covalently linked to the siRNA. The drug can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB. The drug can have a transient effect on the cell. The drug can increase the uptake of the siRNA into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A,

phalloidin, swinholide A, indanocine, or myoservin. The drug can also increase the uptake of the siRNA into a given cell or tissue by activating an inflammatory response, for example. Exemplary drugs that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, a CpG motif, gamma interferon or more generally an agent that activates a toll-like receptor.

siRNA Production

[0470] An siRNA can be produced, *e.g.*, in bulk, by a variety of methods. Exemplary methods include: organic synthesis and RNA cleavage, *e.g.*, *in vitro* cleavage.

[0471] *Organic Synthesis.* An siRNA can be made by separately synthesizing a single stranded RNA molecule, or each respective strand of a double-stranded RNA molecule, after which the component strands can then be annealed.

[0472] A large bioreactor, *e.g.*, the OligoPilot II from Pharmacia Biotec AB (Uppsala Sweden), can be used to produce a large amount of a particular RNA strand for a given siRNA. The OligoPilot II reactor can efficiently couple a nucleotide using only a 1.5 molar excess of a phosphoramidite nucleotide. To make an RNA strand, ribonucleotides amidites are used. Standard cycles of monomer addition can be used to synthesize the 21 to 23 nucleotide strand for the siRNA. Typically, the two complementary strands are produced separately and then annealed, *e.g.*, after release from the solid support and deprotection.

[0473] Organic synthesis can be used to produce a discrete siRNA species. The complementary of the species to a particular target gene can be precisely specified. For example, the species may be complementary to a region that includes a polymorphism, *e.g.*, a single nucleotide polymorphism. Further the location of the polymorphism can be precisely defined. In some embodiments, the polymorphism is located in an internal region, *e.g.*, at least 4, 5, 7, or 9 nucleotides from one or both of the termini.

[0474] *dsiRNA Cleavage.* siRNAs can also be made by cleaving a larger siRNA. The cleavage can be mediated *in vitro* or *in vivo*. For example, to produce iRNAs by cleavage *in vitro*, the following method can be used:

[0475] *In vitro* transcription. dsiRNA is produced by transcribing a nucleic acid (DNA) segment in both directions. For example, the HiScribe™ RNAi transcription kit (New England Biolabs) provides a vector and a method for producing a dsiRNA for a nucleic acid segment that is cloned into the vector at a position flanked on either side by a T7 promoter. Separate templates are generated for T7 transcription of the two complementary strands for

the dsiRNA. The templates are transcribed *in vitro* by addition of T7 RNA polymerase and dsiRNA is produced. Similar methods using PCR and/or other RNA polymerases (*e.g.*, T3 or SP6 polymerase) can also be dotoxins that may contaminate preparations of the recombinant enzymes.

[0476] *In Vitro Cleavage.* In one embodiment, RNA generated by this method is carefully purified to remove endsiRNA is cleaved *in vitro* into siRNAs, for example, using a Dicer or comparable RNAse III-based activity. For example, the dsiRNA can be incubated in an *in vitro* extract from Drosophila or using purified components, *e.g.,* a purified RNAse or RISC complex (RNA-induced silencing complex). See, *e.g.*, Ketting *et al. Genes Dev* 2001 Oct 15;15(20):2654-9; and Hammond *Science* 2001 Aug 10;293(5532):1146-50.

[0477] dsiRNA cleavage generally produces a plurality of siRNA species, each being a particular 21 to 23 nt fragment of a source dsiRNA molecule. For example, siRNAs that include sequences complementary to overlapping regions and adjacent regions of a source dsiRNA molecule may be present.

[0478] Regardless of the method of synthesis, the siRNA preparation can be prepared in a solution (*e.g.*, an aqueous and/or organic solution) that is appropriate for formulation. For example, the siRNA preparation can be precipitated and redissolved in pure double-distilled water, and lyophilized. The dried siRNA can then be resuspended in a solution appropriate for the intended formulation process.

Making iRNA agents conjugated to a targeting ligand

[0479] In some embodiments, the targeting ligand conjugated to the iRNA agent via a nucleobase, sugar moiety, or internucleosidic linkage.

[0480] Conjugation to purine nucleobases or derivatives thereof can occur at any position including, endocyclic and exocyclic atoms. In some embodiments, the 2-, 6-, 7-, or 8-positions of a purine nucleobase are attached to a conjugate moiety. Conjugation to pyrimidine nucleobases or derivatives thereof can also occur at any position. In some embodiments, the 2-, 5-, and 6-positions of a pyrimidine nucleobase can be substituted with a conjugate moiety. When a targeting ligand is conjugated to a nucleobase, the preferred position is one that does not interfere with hybridization, i.e., does not interfere with the hydrogen bonding interactions needed for base pairing. In one embodiment, the targeting ligand may be conjugated to a nucleobase via a linker containing an alkyl, alkenyl or amide linkage.

[0481] Conjugation to sugar moieties of nucleosides can occur at any carbon atom. Exemplary carbon atoms of a sugar moiety that a targeting ligand can be attached to include the 2', 3', and 5' carbon atoms. A targeting ligand can also be attached to the 1' position, such as in an abasic residue. In one embodiment, the targeting ligand may be conjugated to a sugar moiety, via a 2'-O modification, with or without a linker.

[0482] Internucleosidic linkages can also bear targeting ligands. For phosphorus-containing linkages (e.g., phosphodiester, phosphorothioate, phosphorodithioate, phosphoroamidate, and the like), the targeting ligand can be attached directly to the phosphorus atom or to an O, N, or S atom bound to the phosphorus atom. For amine- or amide-containing internucleosidic linkages (e.g., PNA), the targeting ligand can be attached to the nitrogen atom of the amine or amide or to an adjacent carbon atom.

[0483] There are numerous methods for preparing conjugates of oligonucleotides. Generally, an oligonucleotide is attached to a conjugate moiety by contacting a reactive group (e.g., OH, SH, amine, carboxyl, aldehyde, and the like) on the oligonucleotide with a reactive group on the conjugate moiety. In some embodiments, one reactive group is electrophilic and the other is nucleophilic.

[0484] For example, an electrophilic group can be a carbonyl-containing functionality and a nucleophilic group can be an amine or thiol. Methods for conjugation of nucleic acids and related oligonucleotides with and without linking groups are well described in the literature such as, for example, in Manoharan in Antisense Research and Applications, Crooke and LeBleu, eds., CRC Press, Boca Raton, Fla., 1993, Chapter 17, which is incorporated herein by reference in its entirety.

[0485] In one embodiment, a first (complementary) RNA strand and a second (sense) RNA strand can be synthesized separately, wherein one of the RNA strands comprises a pendant targeting ligand, and the first and second RNA strands can be mixed to form a dsRNA. The step of synthesizing the RNA strand preferably involves solid-phase synthesis, wherein individual nucleotides are joined end to end through the formation of internucleotide 3′-5′ phosphodiester bonds in consecutive synthesis cycles.

[0486] In one embodiment, a targeting ligand having a phosphoramidite group is coupled to the 3'-end or 5′-end of either the first (complementary) or second (sense) RNA strand in the last synthesis cycle. In the solid-phase synthesis of an RNA, the nucleotides are initially in the form of nucleoside phosphoramidites. In each synthesis cycle, a further nucleoside phosphoramidite is linked to the -OH group of the previously incorporated nucleotide. If the

targeting ligand has a phosphoramidite group, it can be coupled in a manner similar to a nucleoside phosphoramidite to the free OH end of the RNA synthesized previously in the solid-phase synthesis. The synthesis can take place in an automated and standardized manner using a conventional RNA synthesizer. Synthesis of the targeting ligand having the phosphoramidite group may include phosphitylation of a free hydroxyl to generate the phosphoramidite group.

[0487] In general, the oligonucleotides can be synthesized using protocols known in the art, for example, as described in Caruthers et al., Methods in Enzymology (1992) 211:3-19; WO 99/54459; Wincott et al., Nucl. Acids Res. (1995) 23:2677-2684; Wincott et al., Methods Mol. Bio., (1997) 74:59; Brennan et al., Biotechnol. Bioeng. (1998) 61:33-45; and U.S. Pat. No. 6,001,311; each of which is hereby incorporated by reference in its entirety. In general, the synthesis of oligonucleotides involves conventional nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5′-end, and phosphoramidites at the 3′-end. In a non-limiting example, small scale syntheses are conducted on a Expedite 8909 RNA synthesizer sold by Applied Biosystems, Inc. (Weiterstadt, Germany), using ribonucleoside phosphoramidites sold by ChemGenes Corporation (Ashland, Mass.). Alternatively, syntheses can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, Calif.), or by methods such as those described in Usman et al., J. Am. Chem. Soc. (1987) 109:7845; Scaringe, et al., Nucl. Acids Res. (1990) 18:5433; Wincott, et al., Nucl. Acids Res. (1990) 23:2677-2684; and Wincott, et al., Methods Mol. Bio. (1997) 74:59, each of which is hereby incorporated by reference in its entirety.

[0488] The nucleic acid molecules of the present invention may be synthesized separately and joined together post-synthetically, for example, by ligation (Moore et al., Science (1992) 256:9923; WO 93/23569; Shabarova et al., Nucl. Acids Res. (1991) 19:4247; Bellon et al., Nucleosides & Nucleotides (1997) 16:951; Bellon et al., Bioconjugate Chem. (1997) 8:204; or by hybridization following synthesis and/or deprotection. The nucleic acid molecules can be purified by gel electrophoresis using conventional methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott et al., supra, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

Pharmaceutical Compositions

[0489] In one aspect, the invention features a pharmaceutical composition that includes an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a

larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) including a nucleotide sequence complementary to a target RNA, *e.g.*, substantially and/or exactly complementary. The target RNA can be a transcript of an endogenous human gene. In one embodiment, the siRNA (a) is 19-25 nucleotides long, for example, 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the pharmaceutical composition can be an emulsion, microemulsion, cream, jelly, or liposome.

[0490] In one example the pharmaceutical composition includes an iRNA (an siRNA) mixed with a topical delivery agent. The topical delivery agent can be a plurality of microscopic vesicles. The microscopic vesicles can be liposomes. In some embodiments the liposomes are cationic liposomes.

[0491] In another aspect, the pharmaceutical composition includes an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) admixed with a topical penetration enhancer. In one embodiment, the topical penetration enhancer is a fatty acid. The fatty acid can be arachidonic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester, monoglyceride, diglyceride or pharmaceutically acceptable salt thereof.

[0492] In another embodiment, the topical penetration enhancer is a bile salt. The bile salt can be cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, polyoxyethylene-9-lauryl ether or a pharmaceutically acceptable salt thereof.

[0493] In another embodiment, the penetration enhancer is a chelating agent. The chelating agent can be EDTA, citric acid, a salicyclate, a N-acyl derivative of collagen, laureth-9, an N-amino acyl derivative of a beta-diketone or a mixture thereof.

[0494] In another embodiment, the penetration enhancer is a surfactant, *e.g.*, an ionic or nonionic surfactant. The surfactant can be sodium lauryl sulfate, polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether, a perfluorchemical emulsion or mixture thereof.

[0495] In another embodiment, the penetration enhancer can be selected from a group

consisting of unsaturated cyclic ureas, 1-alkyl-alkones, 1-alkenylazacyclo-alakanones, steroidal anti-inflammatory agents and mixtures thereof. In yet another embodiment the penetration enhancer can be a glycol, a pyrrol, an azone, or a terpenes.

[0496] In one aspect, the invention features a pharmaceutical composition including an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) in a form suitable for oral delivery. In one embodiment, oral delivery can be used to deliver an siRNA composition to a cell or a region of the gastro-intestinal tract, *e.g.*, small intestine, colon (*e.g.*, to treat a colon cancer), and so forth. The oral delivery form can be tablets, capsules or gel capsules. In one embodiment, the siRNA of the pharmaceutical composition modulates expression of a cellular adhesion protein, modulates a rate of cellular proliferation, or has biological activity against eukaryotic pathogens or retroviruses. In another embodiment, the pharmaceutical composition includes an enteric material that substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In some embodiments the enteric material is a coating. The coating can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methylcellulose phthalate or cellulose acetate phthalate.

[0497] In another embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer. The penetration enhancer can be a bile salt or a fatty acid. The bile salt can be ursodeoxycholic acid, chenodeoxycholic acid, and salts thereof. The fatty acid can be capric acid, lauric acid, and salts thereof.

[0498] In another embodiment, the oral dosage form of the pharmaceutical composition includes an excipient. In one example the excipient is polyethyleneglycol. In another example the excipient is precirol.

[0499] In another embodiment, the oral dosage form of the pharmaceutical composition includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate, dibutyl phthalate or triethyl citrate.

[0500] In one aspect, the invention features a pharmaceutical composition including an iRNA (an siRNA) and a delivery vehicle. In one embodiment, the siRNA is (a) is 19-25 nucleotides long, for example, 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nucleotides long.

[0501] In one embodiment, the delivery vehicle can deliver an iRNA (an siRNA), *e.g.*, a

double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) to a cell by a topical route of administration. The delivery vehicle can be microscopic vesicles. In one example the microscopic vesicles are liposomes. In some embodiments the liposomes are cationic liposomes. In another example the microscopic vesicles are micelles. In one aspect, the invention features a pharmaceutical composition including an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) in an injectable dosage form. In one embodiment, the injectable dosage form of the pharmaceutical composition includes sterile aqueous solutions or dispersions and sterile powders. In some embodiments the sterile solution can include a diluent such as water; saline solution; fixed oils, polyethylene glycols, glycerin, or propylene glycol.

[0502] In one aspect, the invention features a pharmaceutical composition including an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) in oral dosage form. In one embodiment, the oral dosage form is selected from the group consisting of tablets, capsules and gel capsules. In another embodiment, the pharmaceutical composition includes an enteric material that substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian stomach. In some embodiments the enteric material is a coating. The coating can be acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate, hydroxy propyl methyl cellulose phthalate or cellulose acetate phthalate. In one embodiment, the oral dosage form of the pharmaceutical composition includes a penetration enhancer, *e.g.*, a penetration enhancer described herein.

[0503] In another embodiment, the oral dosage form of the pharmaceutical composition includes an excipient. In one example the excipient is polyethyleneglycol. In another example the excipient is precirol.

[0504] In another embodiment, the oral dosage form of the pharmaceutical composition includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate, dibutyl phthalate or triethyl citrate.

[0505] In one aspect, the invention features a pharmaceutical composition including an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger

siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) in a rectal dosage form. In one embodiment, the rectal dosage form is an enema. In another embodiment, the rectal dosage form is a suppository.

[0506] In one aspect, the invention features a pharmaceutical composition including an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) in a vaginal dosage form. In one embodiment, the vaginal dosage form is a suppository. In another embodiment, the vaginal dosage form is a foam, cream, or gel.

[0507] In one aspect, the invention features a pharmaceutical composition including an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) in a pulmonary or nasal dosage form. In one embodiment, the siRNA is incorporated into a particle, *e.g.*, a macroparticle, *e.g.*, a microsphere. The particle can be produced by spray drying, lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination thereof. The microsphere can be formulated as a suspension, a powder, or an implantable solid.

Treatment Methods and Routes of Delivery

[0508] Another aspect of the invention relates to a method of reducing the expression of a target gene in a cell, comprising contacting said cell with the oligonucleotide. In one embodiment, the cell is an extrahepatic cell.

[0509] Another aspect of the invention relates to a method of reducing the expression of a target gene in a subject, comprising administering to the subject the oligonucleotide.

[0510] Another aspect of the invention relates to a method of treating a subject having a CNS disorder, comprising administering to the subject a therapeutically effective amount of the double-stranded iRNA agent of the invention, thereby treating the subject. Exemplary CNS disorders that can be treated by the method of the invention include Alzheimer, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, Huntington, Parkinson, spinocerebellar, prion, and lafora.

[0511] The oligonucleotide can be delivered to a subject by a variety of routes, depending on the type of genes targeted and the type of disorders to be treated. In some embodiments,

the oligonucleotide is administered extrahepatically, such as an ocular administration (e.g., intravitreal administration) or an intrathecal or intracerebroventricular administration.

[0512] In one embodiment, the oligonucleotide is administered intrathecally or intracerebroventricularly. By intrathecal or intracerebroventricular administration of the double-stranded iRNA agent, the method can reduce the expression of a target gene in a brain or spine tissue, for instance, cortex, cerebellum, cervical spine, lumbar spine, and thoracic spine.

[0513] In some embodiments, exemplary target genes are APP, ATXN2, C9orf72, TARDBP, MAPT(Tau), HTT, SNCA, FUS, ATXN3, ATXN1, SCA1, SCA7, SCA8, MeCP2, PRNP, SOD1, DMPK, and TTR. To reduce the expression of these target genes in the subject, the oligonucleotide can be administered to the eye(s) directly (e.g., intravitreally). By intravitreal administration of the double-stranded iRNA agent, the method can reduce the expression of the target gene in an ocular tissue.

[0514] For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNAs. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNAs, *e.g.*, unmodified siRNAs, and such practice is within the invention. A composition that includes a iRNA can be delivered to a subject by a variety of routes. Exemplary routes include: intravenous, topical, rectal, anal, vaginal, nasal, pulmonary, ocular.

[0515] The iRNA molecules of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically include one or more species of iRNA and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[0516] The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous

drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular or intracerebroventricular administration.

[0517] The route and site of administration may be chosen to enhance targeting. For example, to target muscle cells, intramuscular injection into the muscles of interest would be a logical choice. Lung cells might be targeted by administering the iRNA in aerosol form. The vascular endothelial cells could be targeted by coating a balloon catheter with the iRNA and mechanically introducing the DNA.

[0518] Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

[0519] Compositions for oral administration include powders or granules, suspensions or solutions in water, syrups, elixirs or non-aqueous media, tablets, capsules, lozenges, or troches. In the case of tablets, carriers that can be used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the nucleic acid compositions can be combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring agents can be added.

[0520] Compositions for intrathecal or intraventricular or intracerebroventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

[0521] Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir. For intravenous use, the total concentration of solutes may be controlled to render the preparation isotonic.

[0522] For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or poly(vinyl alcohol), preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers.

[0523] In one embodiment, the administration of the iRNA (siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, composition is parenteral, *e.g.,* intravenous (*e.g.*, as a bolus or as a diffusible infusion), intradermal, intraperitoneal, intramuscular, intrathecal, intraventricular, intracerebroventricular, intracranial, subcutaneous, transmucosal, buccal, sublingual, endoscopic, rectal, oral, vaginal, topical, pulmonary, intranasal, urethral or ocular. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. Selected modes of delivery are discussed in more detail below.

[0524] *Intrathecal Administration.* In one embodiment, the is delivered by intrathecal injection (i.e. injection into the spinal fluid which bathes the brain and spinal cord tissue). Intrathecal injection of iRNA agents into the spinal fluid can be performed as a bolus injection or via minipumps which can be implanted beneath the skin, providing a regular and constant delivery of siRNA into the spinal fluid. The circulation of the spinal fluid from the choroid plexus, where it is produced, down around the spinal cord and dorsal root ganglia and subsequently up past the cerebellum and over the cortex to the arachnoid granulations, where the fluid can exit the CNS, that, depending upon size, stability, and solubility of the compounds injected, molecules delivered intrathecally could hit targets throughout the entire CNS.

[0525] In some embodiments, the intrathecal administration is via a pump. The pump may be a surgically implanted osmotic pump. In one embodiment, the osmotic pump is implanted into the subarachnoid space of the spinal canal to facilitate intrathecal administration.

[0526] In some embodiments, the intrathecal administration is via an intrathecal delivery system for a pharmaceutical including a reservoir containing a volume of the pharmaceutical agent, and a pump configured to deliver a portion of the pharmaceutical agent contained in the reservoir. More details about this intrathecal delivery system may be found in PCT/US2015/013253, filed on January 28, 2015, which is incorporated by reference in its entirety.

[0527] The amount of intrathecally or intracerebroventricularly injected iRNA agents may vary from one target gene to another target gene and the appropriate amount that has to be applied may have to be determined individually for each target gene. Typically, this amount ranges between 10 μg to 2 mg, preferably 50 μg to 1500 μg, more preferably 100 μg to 1000 μg.

[0528] *Rectal Administration.* The invention also provides methods, compositions, and kits, for rectal administration or delivery of siRNAs described herein.

[0529] Accordingly, an iRNA (an siRNA), *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA , or a DNA which encodes a an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) described herein, *e.g.*, a therapeutically effective amount of a siRNA described herein, *e.g.*, a siRNA having a double stranded region of less than 40, and, for example, less than 30 nucleotides and having one or two 1-3 nucleotide single strand 3' overhangs can be administered rectally, *e.g.*, introduced through the rectum into the lower or upper colon. This approach is particularly useful in the treatment of, inflammatory disorders, disorders characterized by unwanted cell proliferation, *e.g.*, polyps, or colon cancer.

[0530] The medication can be delivered to a site in the colon by introducing a dispensing device, *e.g.*, a flexible, camera-guided device similar to that used for inspection of the colon or removal of polyps, which includes means for delivery of the medication.

[0531] The rectal administration of the siRNA is by means of an enema. The siRNA of the enema can be dissolved in a saline or buffered solution. The rectal administration can also by means of a suppository, which can include other ingredients, e.g., an excipient, e.g., cocoa butter or hydropropylmethylcellulose.

[0532] *Ocular Delivery.* The iRNA agents described herein can be administered to an ocular tissue. For example, the medications can be applied to the surface of the eye or nearby tissue, *e.g.*, the inside of the eyelid. They can be applied topically, *e.g.*, by spraying, in drops, as an eyewash, or an ointment. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. The medication can also be administered to the interior of the eye, and can be introduced by a needle or other delivery device which can introduce it to a selected area or structure. Ocular treatment is particularly desirable for treating inflammation of the eye or nearby tissue.

[0533] In certain embodiments, the double-stranded iRNA agents may be delivered directly to the eye by ocular tissue injection such as periocular, conjunctival, subtenon, intracameral, intravitreal, intraocular, anterior or posterior juxtascleral, subretinal, subconjunctival, retrobulbar, or intracanalicular injections; by direct application to the eye using a catheter or other placement device such as a retinal pellet, intraocular insert, suppository or an implant comprising a porous, non-porous, or gelatinous material; by topical

ocular drops or ointments; or by a slow release device in the cul-de-sac or implanted adjacent to the sclera (transscleral) or in the sclera (intrascleral) or within the eye. Intracameral injection may be through the cornea into the anterior chamber to allow the agent to reach the trabecular meshwork. Intracanalicular injection may be into the venous collector channels draining Schlemm's canal or into Schlemm's canal.

[0534] In one embodiment, the double-stranded iRNA agents may be administered into the eye, for example the vitreous chamber of the eye, by intravitreal injection, such as with pre-filled syringes in ready-to-inject form for use by medical personnel.

[0535] For ophthalmic delivery, the double-stranded iRNA agents may be combined with ophthalmologically acceptable preservatives, co-solvents, surfactants, viscosity enhancers, penetration enhancers, buffers, sodium chloride, or water to form an aqueous, sterile ophthalmic suspension or solution. Solution formulations may be prepared by dissolving the conjugate in a physiologically acceptable isotonic aqueous buffer. Further, the solution may include an acceptable surfactant to assist in dissolving the double-stranded iRNA agents. Viscosity building agents, such as hydroxymethyl cellulose, hydroxyethyl cellulose, methylcellulose, polyvinylpyrrolidone, or the like may be added to the pharmaceutical compositions to improve the retention of the double-stranded iRNA agents.

[0536] To prepare a sterile ophthalmic ointment formulation, the double-stranded iRNA agents is combined with a preservative in an appropriate vehicle, such as mineral oil, liquid lanolin, or white petrolatum. Sterile ophthalmic gel formulations may be prepared by suspending the double-stranded iRNA agents in a hydrophilic base prepared from the combination of, for example, CARBOPOL®-940 (BF Goodrich, Charlotte, N.C.), or the like, according to methods known in the art.

[0537] *Topical Delivery.* Any of the siRNAs described herein can be administered directly to the skin. For example, the medication can be applied topically or delivered in a layer of the skin, *e.g.*, by the use of a microneedle or a battery of microneedles which penetrate into the skin, but, for example, not into the underlying muscle tissue. Administration of the siRNA composition can be topical. Topical applications can, for example, deliver the composition to the dermis or epidermis of a subject. Topical administration can be in the form of transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids or powders. A composition for topical administration can be formulated as a liposome, micelle, emulsion, or other lipophilic molecular assembly. The transdermal administration can be applied with at least one penetration enhancer, such as

iontophoresis, phonophoresis, and sonophoresis.

[0538] *For* ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNAs. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNAs, *e.g.*, unmodified siRNAs, and such practice is within the invention. In some embodiments, an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) is delivered to a subject via topical administration. "Topical administration" refers to the delivery to a subject by contacting the formulation directly to a surface of the subject. The most common form of topical delivery is to the skin, but a composition disclosed herein can also be directly applied to other surfaces of the body, *e.g.*, to the eye, a mucous membrane, to surfaces of a body cavity or to an internal surface. As mentioned above, the most common topical delivery is to the skin. The term encompasses several routes of administration including, but not limited to, topical and transdermal. These modes of administration typically include penetration of the skin's permeability barrier and efficient delivery to the target tissue or stratum. Topical administration can be used as a means to penetrate the epidermis and dermis and ultimately achieve systemic delivery of the composition. Topical administration can also be used as a means to selectively deliver oligonucleotides to the epidermis or dermis of a subject, or to specific strata thereof, or to an underlying tissue.

[0539] The term "skin," as used herein, refers to the epidermis and/or dermis of an animal. Mammalian skin consists of two major, distinct layers. The outer layer of the skin is called the epidermis. The epidermis is comprised of the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale, with the stratum corneum being at the surface of the skin and the stratum basale being the deepest portion of the epidermis. The epidermis is between 50 μm and 0.2 mm thick, depending on its location on the body.

[0540] Beneath the epidermis is the dermis, which is significantly thicker than the epidermis. The dermis is primarily composed of collagen in the form of fibrous bundles. The collagenous bundles provide support for, inter alia, blood vessels, lymph capillaries, glands, nerve endings and immunologically active cells.

[0541] One of the major functions of the skin as an organ is to regulate the entry of substances into the body. The principal permeability barrier of the skin is provided by the stratum corneum, which is formed from many layers of cells in various states of

differentiation. The spaces between cells in the stratum corneum is filled with different lipids arranged in lattice-like formations that provide seals to further enhance the skins permeability barrier.

[0542] The permeability barrier provided by the skin is such that it is largely impermeable to molecules having molecular weight greater than about 750 Da. For larger molecules to cross the skin's permeability barrier, mechanisms other than normal osmosis must be used.

[0543] Several factors determine the permeability of the skin to administered agents. These factors include the characteristics of the treated skin, the characteristics of the delivery agent, interactions between both the drug and delivery agent and the drug and skin, the dosage of the drug applied, the form of treatment, and the post treatment regimen. To selectively target the epidermis and dermis, it is sometimes possible to formulate a composition that comprises one or more penetration enhancers that will enable penetration of the drug to a preselected stratum.

[0544] Transdermal delivery is a valuable route for the administration of lipid soluble therapeutics. The dermis is more permeable than the epidermis and therefore absorption is much more rapid through abraded, burned or denuded skin. Inflammation and other physiologic conditions that increase blood flow to the skin also enhance transdermal adsorption. Absorption via this route may be enhanced by the use of an oily vehicle (inunction) or through the use of one or more penetration enhancers. Other effective ways to deliver a composition disclosed herein via the transdermal route include hydration of the skin and the use of controlled release topical patches. The transdermal route provides a potentially effective means to deliver a composition disclosed herein for systemic and/or local therapy.

[0545] In addition, iontophoresis (transfer of ionic solutes through biological membranes under the influence of an electric field) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 163), phonophoresis or sonophoresis (use of ultrasound to enhance the absorption of various therapeutic agents across biological membranes, notably the skin and the cornea) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 166), and optimization of vehicle characteristics relative to dose position and retention at the site of administration (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 168) may be useful methods for enhancing the transport of topically applied compositions across skin and mucosal sites.

[0546] The compositions and methods provided may also be used to examine the function of various proteins and genes *in vitro* in cultured or preserved dermal tissues and in animals. The invention can be thus applied to examine the function of any gene. The methods of the invention can also be used therapeutically or prophylactically. For example, for the treatment of animals that are known or suspected to suffer from diseases such as psoriasis, lichen planus, toxic epidermal necrolysis, ertythema multiforme, basal cell carcinoma, squamous cell carcinoma, malignant melanoma, Paget's disease, Kaposi's sarcoma, pulmonary fibrosis, Lyme disease and viral, fungal and bacterial infections of the skin.

[0547] *Pulmonary Delivery.* Any of the siRNAs described herein can be administered to the pulmonary system. Pulmonary administration can be achieved by inhalation or by the introduction of a delivery device into the pulmonary system, *e.g.*, by introducing a delivery device which can dispense the medication. Certain embodiments may use a method of pulmonary delivery by inhalation. The medication can be provided in a dispenser which delivers the medication, *e.g.*, wet or dry, in a form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication. Pulmonary delivery is effective not only for disorders which directly affect pulmonary tissue, but also for disorders which affect other tissue. siRNAs can be formulated as a liquid or nonliquid, *e.g.*, a powder, crystal, or aerosol for pulmonary delivery.

[0548] For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNAs. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNAs, *e.g.*, unmodified siRNAs, and such practice is within the invention. A composition that includes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, (*e.g.*, a precursor, *e.g.*, a larger siRNA which can be processed into a ssiRNA, or a DNA which encodes an siRNA, *e.g.*, a double-stranded siRNA, or ssiRNA, or precursor thereof) can be administered to a subject by pulmonary delivery. Pulmonary delivery compositions can be delivered by inhalation by the patient of a dispersion so that the composition, for example, iRNA, within the dispersion can reach the lung where it can be readily absorbed through the alveolar region directly into blood circulation. Pulmonary delivery can be effective both for systemic delivery and for localized delivery to treat diseases of the lungs.

[0549] Pulmonary delivery can be achieved by different approaches, including the use of nebulized, aerosolized, micellular and dry powder-based formulations. Delivery can be

achieved with liquid nebulizers, aerosol-based inhalers, and dry powder dispersion devices. Metered-dose devices are may be used. One of the benefits of using an atomizer or inhaler is that the potential for contamination is minimized because the devices are self contained. Dry powder dispersion devices, for example, deliver drugs that may be readily formulated as dry powders. A iRNA composition may be stably stored as lyophilized or spray-dried powders by itself or in combination with suitable powder carriers. The delivery of a composition for inhalation can be mediated by a dosing timing element which can include a timer, a dose counter, time measuring device, or a time indicator which when incorporated into the device enables dose tracking, compliance monitoring, and/or dose triggering to a patient during administration of the aerosol medicament.

[0550] The term "powder" means a composition that consists of finely dispersed solid particles that are free flowing and capable of being readily dispersed in an inhalation device and subsequently inhaled by a subject so that the particles reach the lungs to permit penetration into the alveoli. Thus, the powder is said to be "respirable." For example, the average particle size is less than about 10 μm in diameter with a relatively uniform spheroidal shape distribution. In some embodiments, the diameter is less than about 7.5 μm and in some embodiments less than about 5.0 μm. Usually the particle size distribution is between about 0.1 μm and about 5 μm in diameter, sometimes about 0.3 μm to about 5 μm.

[0551] The term "dry" means that the composition has a moisture content below about 10% by weight (% w) water, usually below about 5% w and in some cases less it than about 3% w. A dry composition can be such that the particles are readily dispersible in an inhalation device to form an aerosol.

[0552] The term "therapeutically effective amount" is the amount present in the composition that is needed to provide the desired level of drug in the subject to be treated to give the anticipated physiological response.

[0553] The term "physiologically effective amount" is that amount delivered to a subject to give the desired palliative or curative effect.

[0554] The term "pharmaceutically acceptable carrier" means that the carrier can be taken into the lungs with no significant adverse toxicological effects on the lungs.

[0555] The types of pharmaceutical excipients that are useful as carrier include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

[0556] Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A group of carbohydrates may include lactose, threhalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame. Amino acids include alanine and glycine, with glycine being used in some embodiments.

[0557] Additives, which are minor components of the composition of this invention, may be included for conformational stability during spray drying and for improving dispersibility of the powder. These additives include hydrophobic amino acids such as tryptophan, tyrosine, leucine, phenylalanine, and the like.

[0558] Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate may be used in some embodiments.

[0559] Pulmonary administration of a micellar iRNA formulation may be achieved through metered dose spray devices with propellants such as tetrafluoroethane, heptafluoroethane, dimethylfluoropropane, tetrafluoropropane, butane, isobutane, dimethyl ether and other non-CFC and CFC propellants.

[0560] *Oral or Nasal Delivery.* Any of the siRNAs described herein can be administered orally, e.g., in the form of tablets, capsules, gel capsules, lozenges, troches or liquid syrups. Further, the composition can be applied topically to a surface of the oral cavity.

[0561] Any of the siRNAs described herein can be administered nasally. Nasal administration can be achieved by introduction of a delivery device into the nose, *e.g.*, by introducing a delivery device which can dispense the medication. Methods of nasal delivery include spray, aerosol, liquid, *e.g.*, by drops, or by topical administration to a surface of the nasal cavity. The medication can be provided in a dispenser with delivery of the medication, *e.g.*, wet or dry, in a form sufficiently small such that it can be inhaled. The device can deliver a metered dose of medication. The subject, or another person, can administer the medication.

[0562] Nasal delivery is effective not only for disorders which directly affect nasal tissue, but also for disorders which affect other tissue siRNAs can be formulated as a liquid or nonliquid, *e.g.*, a powder, crystal, or for nasal delivery. As used herein, the term “crystalline”

describes a solid having the structure or characteristics of a crystal, *i.e.*, particles of three-dimensional structure in which the plane faces intersect at definite angles and in which there is a regular internal structure. The compositions of the invention may have different crystalline forms. Crystalline forms can be prepared by a variety of methods, including, for example, spray drying.

[0563] For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNAs. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNAs, *e.g.*, unmodified siRNAs, and such practice is within the invention. Both the oral and nasal membranes offer advantages over other routes of administration. For example, drugs administered through these membranes have a rapid onset of action, provide therapeutic plasma levels, avoid first pass effect of hepatic metabolism, and avoid exposure of the drug to the hostile gastrointestinal (GI) environment. Additional advantages include easy access to the membrane sites so that the drug can be applied, localized and removed easily.

[0564] In oral delivery, compositions can be targeted to a surface of the oral cavity, *e.g.*, to sublingual mucosa which includes the membrane of ventral surface of the tongue and the floor of the mouth or the buccal mucosa which constitutes the lining of the cheek. The sublingual mucosa is relatively permeable thus giving rapid absorption and acceptable bioavailability of many drugs. Further, the sublingual mucosa is convenient, acceptable and easily accessible.

[0565] The ability of molecules to permeate through the oral mucosa appears to be related to molecular size, lipid solubility and peptide protein ionization. Small molecules, less than 1000 daltons appear to cross mucosa rapidly. As molecular size increases, the permeability decreases rapidly. Lipid soluble compounds are more permeable than non-lipid soluble molecules. Maximum absorption occurs when molecules are un-ionized or neutral in electrical charges. Therefore charged molecules present the biggest challenges to absorption through the oral mucosae.

[0566] A pharmaceutical composition of iRNA may also be administered to the buccal cavity of a human being by spraying into the cavity, without inhalation, from a metered dose spray dispenser, a mixed micellar pharmaceutical formulation as described above and a propellant. In one embodiment, the dispenser is first shaken prior to spraying the pharmaceutical formulation and propellant into the buccal cavity. For example, the medication can be sprayed into the buccal cavity or applied directly, *e.g.*, in a liquid, solid, or

gel form to a surface in the buccal cavity. This administration is particularly desirable for the treatment of inflammations of the buccal cavity, *e.g.*, the gums or tongue, *e.g.*, in one embodiment, the buccal administration is by spraying into the cavity, *e.g.*, without inhalation, from a dispenser, *e.g.*, a metered dose spray dispenser that dispenses the pharmaceutical composition and a propellant.

[0567] An aspect of the invention also relates to a method of delivering an oligonucleotide into the CNS by intrathecal or intracerebroventricular delivery, or into an ocular tissue by ocular delivery, e.g., an intravitreal delivery.

[0568] Some embodiments relates to a method of reducing the expression of a target gene in a subject, comprising administering to the subject the oligonucleotide described herein. In one embodiment, the oligonucleotide is administered intrathecally or intracerebroventricularly (to reduce the expression of a target gene in a brain or spine tissue). In one embodiment, the oligonucleotide is administered ocularly, e.g., intravitreally, (to reduce the expression of a target gene in an ocular tissue).

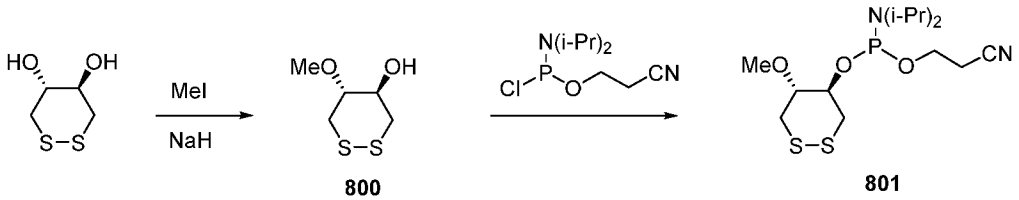
[0569] The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

[0570] The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

Example 1. Synthesis of cyclic disulfide modified phosphate prodrug derivatives

Scheme 1

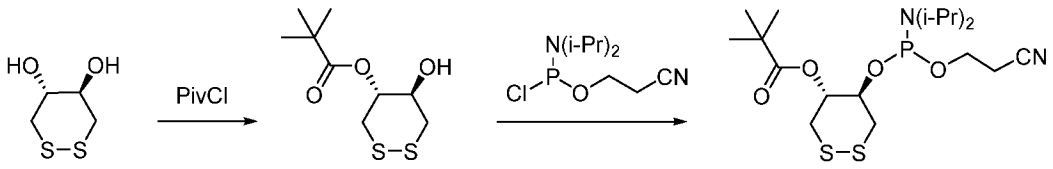


[0571] **Compound 800:** *Trans*-1,2-dithiane-4,5-diol (3.04 g, 20 mmol), was dissolved in anhydrous THF (30 mL) under inert atmosphere and cooled in a water/ice bath. Sodium hydride, 60% dispersion in oil (0.84 g, 21 mmol) was added and the mixture was stirred for

30 minutes. Iodomethane (3.7 mL, 60 mmol) was added and the mixture was allowed to slowly warm to room temperature overnight. The reaction mixture was concentrated under vacuum to a colorless liquid. The product was isolated by silica gel flash chromatography of crude (3.66 g) with isocratic 30% ethyl acetate in hexane (1:9 to 1:1 gradient). Obtained 1.11 g (33%) of **800** as yellowish oil. ^{1}H NMR, (400 MHz, DMSO-d_6) δ 5.29 (d, J = 4.8 Hz, 1H); 3.44 (septet, J = 4.8 Hz, 1H); 3.30 (dd, J = 3.6, 13.2 Hz, 1H); 3.11-3.03 (m, 2H); 2.74 (dd, J = 10.0, 13.6 Hz, 1H); 2.67 (dd, J = 10.0, 13.6 Hz, 1H).

[0572] **Compound 801:** 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.80 mL, 8 mmol) was added to a stirred solution of carbinol **800** (1.07 g, 6.4 mmol) and DIEA (1.40 mL, 8 mmol) in anhydrous ethyl acetate (30 mL) under Ar atmosphere. The mixture was stirred at room temperature for 1 hour and quenched. The organic phase was separated, washed twice with 5% NaCl, saturated NaCl, dried over anhydrous sodium sulfate, and the crude residue was purified over a column of silica gel with isocratic 25% ethyl acetate containing 1% of TEA in hexane to afford 1.88 g (80%) of pure amidite **801** as a yellowish oil. ^{1}H NMR (400 MHz, CD_3CN): δ 3.94-3.74 (m, 2.5H); 3.74-3.55 (m, 2.5H); 3.39 (s, 1.5H); 3.38 (s, 1.5H); 3.36-3.15 (m, 3H); 2.91 (dd, J = 9.2, 13.6 Hz, 1H); 2.85-2.77 (m, 1H); 2.72-2.59 (m, 2H); 1.24-1.13 (m, 12H). ^{13}C NMR (101 MHz, CD_3CN) δ 119.64; 119.61; 59.84; 59.67; 59.11; 58.91; 58.38; 58.10; 57.64; 44.10; 43.98; 25.03; 24.96; 24.95; 24.92; 24.90; 24.84; 24.76; 21.09; 21.03. ^{31}P NMR (202 MHz, CD_3CN): δ 150.68; 150.37.

Scheme 2



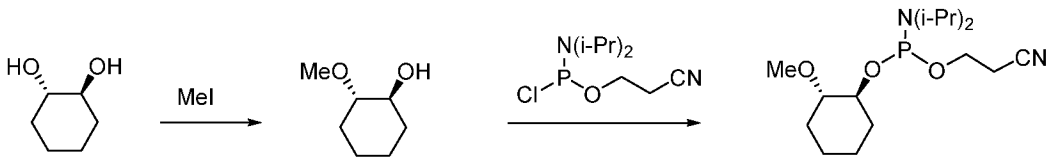
802 803

[0573] **Compound 802:** *Trans*-1,2-dithiane-4,5-diol (5.0 g, 32.8 mmol), was dissolved in pyridine (160 mL) under inert atmosphere and cooled in a water/ice bath. Trimethylacetyl chloride (14.2 mL, 98.5 mmol) was added over 5 minutes, the mixture was warmed to room temperature and stirred for 1.5 hours. The mixture was concentrated under vacuum, re-dissolved in ethyl acetate (100 mL), washed with 5% NaCl (2 x 100 mL), saturated NaCl (1 x 100 mL), dried over Na_2SO_4, filtered, and concentrated to an oil. The product was purified by silica gel flash chromatography, 120 g silica column, with ethyl acetate:hexane (1:4 to 1:2 gradient). The product-containing fractions were concentrated, chased with acetonitrile (2x),

and dried under high vacuum. **Compound 802** was obtained as a white solid, 66% yield (5.12 g). ^{1}H NMR, (500 MHz, DMSO-d_6) δ 5.43 (d, J = 5.9 Hz, 1H), 4.67 – 4.60 (m, 1H), 3.64 – 3.55 (m, 1H), 3.14 (dd, J = 13.2, 3.9 Hz, 2H), 2.90 – 2.80 (m, 2H), 1.15 (s, 9H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 176.54, 38.28, 26.79.

[0574] **Compound 803: Compound 802** (3.0 g, 12.7 mmol) was dissolved in anhydrous ethyl acetate (60 mL) under an inert atmosphere. *N,N*-diisopropylethylamine (2.9 mL, 16.5 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (3.7 mL, 16.5 mmol) were added and the mixture was stirred at room temperature for 2 hours. The reaction mixture was quenched, washed with 5% NaCl (3 x 200 mL), saturated NaCl (1 x 100 mL), dried over anhyd. Na_2SO_4, filtered, and concentrated. The product was purified by silica gel flash chromatography, 80 g silica column, using isocratic ethyl acetate (+ 0.5 % triethylamine):hexane (1:10). The product-containing fractions were concentrated in vacuum, chased with acetonitrile (2x), and dried in high vacuum. **Compound 803** was isolated as a colorless oil, 77 % yield (4.24 g). ^{1}H NMR, (500 MHz, Acetonitrile-d_3) δ 4.88 – 4.75 (m, 1H), 4.03 – 3.92 (m, 1H), 3.89 – 3.68 (m, 2H), 3.67 – 3.56 (m, 2H), 3.46 – 3.32 (m, 1H), 3.03 – 2.84 (m, 2H), 2.71 – 2.59 (m, 2H), 1.25 – 1.11 (m, 21H). ^{13}C NMR (101 MHz, Acetonitrile-d_3) δ 178.02, 119.47, 59.71, 59.51, 59.25, 59.05, 44.29, 44.16, 44.09, 43.96, 39.59, 27.55, 27.52, 25.10, 25.04, 25.03, 24.96, 24.90, 24.82, 21.13, 21.10, 21.06, 21.03. ^{31}P NMR (202 MHz, Acetonitrile-d_3) δ 151.22, 148.72.

Scheme 3



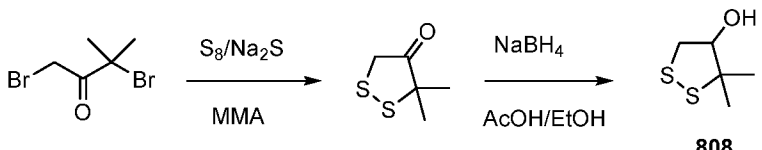
804 805

[0575] **Compound 804:** *Trans*-1,2-cyclohexanediol (10.1 g, 87.0 mmol), was dissolved in THF (120 mL) under inert atmosphere and cooled in a water/ice bath. Sodium hydride, 60% dispersion in oil (3.96 g, 91.4 mmol) was added and the reaction was stirred for 1.5 hours. Iodomethane (16.3 mL, 261.1 mmol) was added and the reaction was allowed to slowly warm to room temperature over 17 hours. The reaction mixture was concentrated under vacuum to a colorless liquid. The product was isolated by silica gel flash chromatography, 220 g silica column, using ethyl acetate:hexane (1:9 to 1:1 gradient). The product-containing fractions were concentrated and chased with dichloromethane (2x).

Compound 804 was obtained as a colorless oil, 14% yield (1.6 g). ^{1}H NMR, (400 MHz, DMSO-d_6) δ 4.57 (d, J = 4.2 Hz, 1H), 3.30 – 3.22 (m, 4H), 2.89 – 2.79 (m, 1H), 1.93 – 1.85 (m, 1H), 1.77 – 1.66 (m, 1H), 1.62 – 1.48 (m, 2H), 1.17 – 1.00 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 83.29, 71.52, 56.35, 32.64, 28.30, 23.14, 23.03.

[0576] **Compound 805:** Compound **804** (0.51 g, 3.9 mmol) was dissolved in anhydrous ethyl acetate (20 mL) under inert atmosphere. *N,N*-diisopropylethylamine (1.0 mL, 5.9 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (1.3 mL, 5.9 mmol) were added, and the mixture was stirred at room temperature for 3 hours. The mixture was quenched and diluted with ethyl acetate (60 mL). The organic phase was separated, washed with 5% NaCl (3 x 150 mL), saturated NaCl (1 x 150 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuum. The product was purified by silica gel flash chromatography over a 24 g silica column quenched with triethylamine (10 mL) using ethyl acetate:hexane (1:9 to 1:2 gradient). The product-containing fractions were concentrated, chased with acetonitrile (2x), and dried under high vacuum. Compound **805** was obtained as a colorless oil, 79% yield (1.02 g). ^{1}H NMR, (400 MHz, Acetonitrile-d_3) δ 3.87 – 3.54 (m, 5H), 3.32 (d, J = 2.4 Hz, 3H), 3.14 – 3.02 (m, 1H), 2.70 – 2.57 (m, 2H), 2.02 – 1.83 (m, 2H), 1.66 – 1.55 (m, 2H), 1.47 – 1.21 (m, 4H), 1.21 – 1.13 (m, 12H). ^{13}C NMR (126 MHz, Acetonitrile-d_3) δ 83.00 , 82.75 , 76.03 , 75.55 , 59.64 , 59.50 , 59.13 , 58.98 , 57.46 , 56.97 , 44.05 , 44.03 , 43.95 , 43.93 , 32.77 , 32.42 , 29.34 , 29.17 , 25.07 , 25.05 , 25.01 , 25.00 , 24.87 , 24.83 , 24.81 , 24.77 , 23.89 , 23.76 , 23.63 , 23.60 , 21.19 , 21.13 , 21.08 . ^{31}P NMR (162 MHz, Acetonitrile-d_3) δ 148.85, 148.56.

Scheme 4



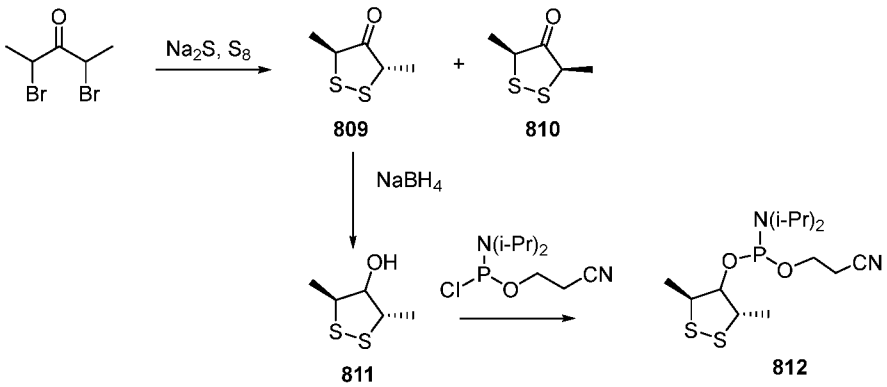
806 **807** **808**

[0577] **Compound 807:** A suspension of sodium sulfide nonahydrate (4.08 g, 17 mmol) and elemental sulfur (1.09 g, 34 mmol) in MMA (N-methylacetamide) (35 mL) was stirred at 30 °C overnight to form homogeneous yellowish solution. A solution of dibromoketone **806** (3.45 g, 14 mmol) in MMA (10 mL) was added dropwise for ~ 30 minutes while maintaining bath temperature at 30 °C. The mixture was stirred at 30 °C for additional 2 hours, cooled to room temperature, and quenched by addition of 5% aqeous NaCl (200 mL). The mixture was extracted with ethyl acetate, and the organic phase was separated, washed with 5% aqeous

NaCl, saturated NaCl, and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuum to afford crude residue (2.17 g) that was purified over a column of silica gel with 5% of ethyl acetate in hexane to afford 0.97 g (47%) of pure dimethyl ketone **807**. ^{1}H NMR (400 MHz, CDCl3): δ 3.58 (s, 2H); 1.52 (s, 6H). ^{13}C NMR (126 MHz, CDCl3): δ 210.5; 55.8; 41.7; 23.7.

[0578] **Compound 808:** Sodium borohydride (122 mg, 3.2 mmol) was added to a cooled (-78 °C) and stirred solution of ketone **807** (0.94 g, 6.4 mmol) and acetic acid (0.37 mL, 6.4 mmol) in dry ethanol (15 mL) under Ar atmosphere. The mixture was stirred at -78 °C for 2 hours, the cooling bath was removed, and the mixture was quenched by addition of saturated ammonium chloride (15 mL) and ethyl acetate (20 mL). The mixture was allowed to warm up to room temperature and water (8 mL) was added to dissolve solids. The organic phase was separated, washed consecutively with 15% aqeous NaCl, saturated sodium bicarbonate, saturated NaCl, and dried over anhydrous sodium sulfate. The solvent was removed in vacuum to afford crude product (0.93 g) that was purified over a column of silica gel with gradient of 10 to 30% of Ethyl acetate in hexane to afford 0.66 g (70%) of **808** as slowly crystallizing yellowish oil. ^{1}H NMR (500 MHz, CD_3CN): δ 4.10-4.05 (m, 1H); 3.39 (dd, J = 5.5, 11.0 Hz, 1H); 3.17 (d, J = 8 Hz, 1H); 3.03 (dd, J = 4.0, 11.0 Hz, 1H); 1.41 (s, 3H); 1.37 (s, 3H). C13 NMR (126 MHz, CDCl3): δ 82.7; 65.0; 43.4; 26.6; 21.4.

Scheme 5



[0579] **Compound 809 and 810:** N-Methylacetamide (100 mL) was heated to 30 °C under inert atmosphere. Disodiumsulfide-nonahydrate (8.38 g, 34.8 mmol) and sulfur (2.24 g, 69.7 mmol) were added and the suspension was stirred at 35 °C for 24 hours to dissolve solids. Solution of 2,4-dibromo-3-pentanone (8.47 g, 34.8 mmol) in N-methylacetamine (10 mL) and was added slowly over 20 minutes. The mixture was stirred at 30 °C for 20 hours and quenched by slowly pouring to a stirred solution of 5% NaCl (400 mL). The mixture was

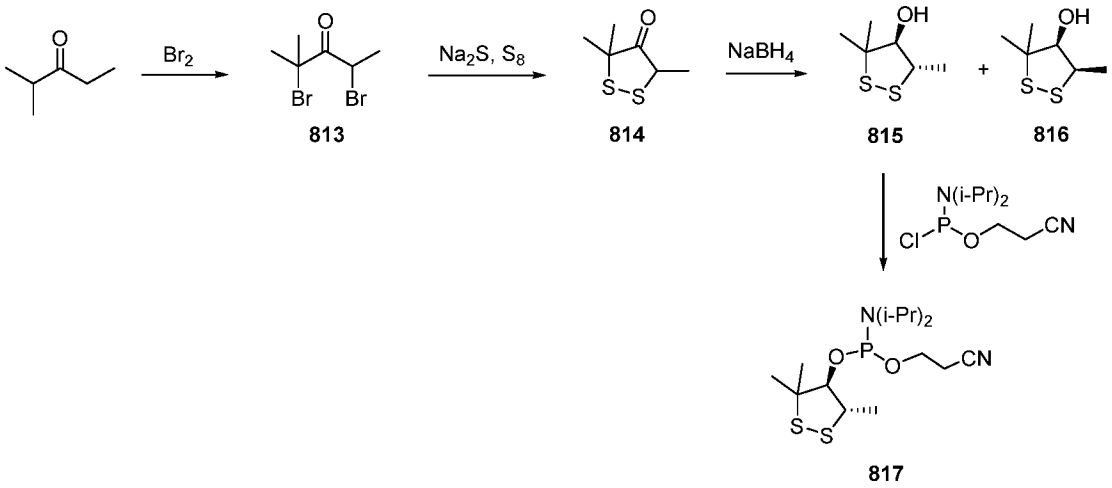
diluted with ethyl acetate (400 mL), and the organic layer was separated, washed with 5% NaCl (1 x 300 mL), saturated NaCl (1 x 300 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated. Oily residue was diluted in hexane (200 mL) and stirred for 18 hours. Solids were removed by filtration and the filtrate was concentrated to an oil. The product was purified by silica gel flash chromatography, 120 g silica column, using ethyl acetate:hexane (0 to 10% gradient). Early eluting compound **809** was isolated as a yellow liquid, 31% yield (1.31 g). Later eluting compound **810** was isolated as a yellow oil, 9% yield (0.38 g, 4:1 mix of **810** to **809**). **Compound 809:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.84 (q, J = 6.9 Hz, 2H), 1.35 (d, J = 6.9 Hz, 6H). **Compound 810:** ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.93 (q, J = 7.0 Hz, 2H), 1.37 (d, J = 7.0 Hz, 6H).

[0580] **Compound 811:** Ketone **809** (1.02 g, 6.75 mmol) was dissolved in ethanol (15 mL) under inert atmosphere and cooled to -78 °C. Acetic acid (0.39 mL, 6.75 mmol) was added, followed by sodium borohydride (130 mg, 3.37 mmol). The mixture was stirred for 5 hours, and a second portion of sodium borohydride (130 mg, 3.37 mmol) was added. The mixture was stirred for additional 2 hours, quenched with saturated NH_4Cl (10 mL), and allowed to warm to room temperature. Ethyl acetate (20 mL), saturated NH_4Cl (10 mL), and water (10 mL) were added, and the mixture was stirred at room temperature overnight. The organic layer was separated, washed with 1:1 saturated NH_4Cl:water (1 x 20 mL), saturated $NaHCO_3$ (1 x 25 mL), and saturated NaCl (1 x 25 mL), dried over Na_2SO_4, filtered, and concentrated. The product was purified by silica gel flash chromatography, 24 g silica column, using ethyl acetate:hexane (1:15 to 1:9 gradient). The product-containing fractions were concentrated, chased with dichloromethane (2x), and dried under high vacuum overnight. **Compound 811** was obtained as a colorless oil, 42% yield (427 mg). ^{1}H NMR, (400 MHz, DMSO-d_6) δ 5.35 (d, J = 5.6 Hz, 1H), 3.93 (q, J = 5.1 Hz, 1H), 3.62 – 3.53 (m, 1H), 3.44 – 3.35 (m, 1H), 1.28 (t, J = 6.9 Hz, 6H).

[0581] **Compound 812:** Compound **811** (0.40 g, 2.66 mmol) was dissolved in anhydrous ethyl acetate (13 mL) under inert atmosphere. *N,N*-diisopropylethylamine (0.70 mL, 4.0 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.95 mL, 4.0 mmol) were added, and the mixture was stirred at room temperature for 1.5 hours. The mixture was quenched, washed with 5% NaCl (3 x 40 mL), saturated NaCl (1 x 40 mL), dried over Na_2SO_4, filtered, and concentrated. The product was purified by silica gel flash chromatography, 24 g silica column, using ethyl acetate (+ 1 % triethylamine):hexane (1:15 to 1:9 gradient). The product-containing fractions were concentrated under vacuum, chased

with acetonitrile (2x), and dried under high vacuum. **Compound 812** was isolated as a yellow oil, 20 % yield (182 mg). ^{1}H NMR, (500 MHz, Acetonitrile-d_3) δ 4.28 – 4.19 (m, 1H), 3.89 – 3.57 (m, 6H), 2.73 – 2.61 (m, 2H), 1.42 – 1.35 (m, 6H), 1.22 – 1.16 (m, 12H). ^{31}P NMR (202 MHz, Acetonitrile-d_3) δ 150.85, 150.47.

Scheme 6



[0582] **Compound 813:** 2-Methyl-3-pentanone (23.3 g, 233 mmol) was dissolved in diethyl ether (100mL) under inert atmosphere. A separately prepared solution of bromine (25.7 mL, 465 mmol) in dichloromethane (50 mL) (12 drops) was added to the ketone solution and stirred for 1 minute to initiate the reaction. The mixture was cooled in an ice/water bath, and the bromine solution (65 mL) was added dropwise to cooled and stirred ketone solution over 3 hours. The ice bath was removed, and the mixture was stirred at room temperature for 20 minutes. The mixture was diluted with diethyl ether (300 mL) and added portion-wise to a stirred aqueous solution of 5% NaCl (300 mL) and stirred 10 minutes. The organic layer was washed with 5% NaCl (2 x 500 mL), 5% $Na_2S_2O_5$ (1 x 450 mL) and saturated NaCl (1 x 500 mL), dried over Na_2SO_4, filtered, and concentrated. **Compound 813** was isolated as a light-yellow liquid, 95% yield (57.1 g). ^{1}H NMR, (500 MHz, DMSO-d_6) δ 5.41 (q, J = 6.6 Hz, 1H), 1.98 (s, 3H), 1.87 (s, 3H), 1.74 (d, J = 6.6 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 198.94, 64.58, 41.06, 29.35, 28.59, 22.10.

[0583] **Compound 814:** N-Methylacetamide (300 mL) was heated at 33 °C under inert atmosphere. Disodiumsulfide-nonahydrate (27.9 g, 116 mmol) and sulfur (7.46 g, 233 mmol) were added and the suspension was stirred at 35 °C for 24 hours to dissolve solids. The reaction was cooled to 30 °C and a solution of **Compound 813** (30 g, 116 mmol) in N-methylacetamide (20 mL) was added slowly over 15 minutes. The mixture was stirred at 30

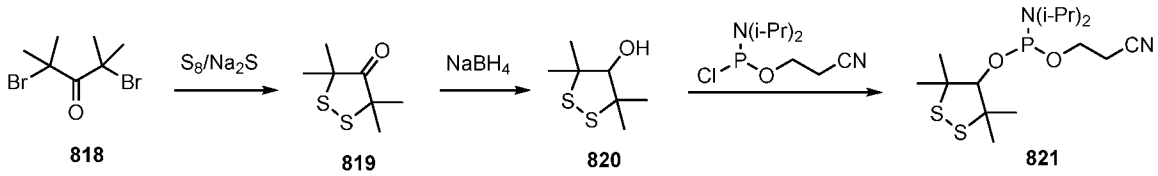
°C for 22 hours and quenched by slowly pouring to a stirred solution of 5% NaCl (1200 mL). The mixture was diluted with ethyl acetate (1200 mL), and washed with 5% NaCl (3 x 1200 mL) and saturated NaCl (1 x 800 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The oily residue was diluted in hexane (600 mL) and stirred for 18 hours. Solids were removed by decanting and the supernatant was concentrated to an oil. The product was purified by silica gel flash chromatography, 220 g silica column, using dichloromethane:hexane (1:9 to 1:8 gradient). The product-containing fractions were concentrated and chased with dichloromethane (2x). **Compound 814** was isolated as a yellow oil, 32% yield (6.1 g). ^{1}H NMR, ELN0021-16-7 (400 MHz, DMSO-d_6) δ 3.98 (q, J = 7.0 Hz, 1H), 1.45 (d, J = 9.0 Hz, 6H), 1.37 (d, J = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 211.58, 56.27, 50.15, 24.69, 24.11, 16.00.

[0584] **Compound 815 and 816:** Compound **814** (2.3 g, 14.2 mmol) was dissolved in ethanol (35 mL) under inert atmosphere and cooled to -78 °C. Sodium borohydride (531 mg, 4.17 mmol) was added, and the reaction mixture was stirred for 15 minutes, warmed to room temperature, and stirred for additional 3 hours. The mixture was cooled to -78 °C and quenched with saturated NH_4Cl (10 mL). Ethyl acetate (50 mL), saturated NH_4Cl (35 mL), and water (20 mL) were added and the mixture was stirred at room temperature overnight. The organic layer was separated, washed with 1:1 saturated NH_4Cl:water (1 x 50 mL), saturated $NaHCO_3$ (1 x 50 mL), saturated NaCl (1 x 50 mL), dried over Na_2SO_4, filtered, and concentrated. The product was isolated by silica gel flash chromatography, 80 g silica column, using ethyl acetate:hexane (1:20 to 1:9 gradient). The product-containing fractions were concentrated and chased with dichloromethane (2x). Early eluting **Compound 815** was isolated as a yellow solid, 40% yield (0.93 g). Later eluting **Compound 816** was isolated as a yellow oil, 10% yield (0.23 g). **Compound 815:** ^{1}H NMR, (500 MHz, DMSO-d_6) δ 5.13 (d, J = 6.9 Hz, 1H), 3.88 – 3.82 (m, 1H), 3.76 (dd, J = 6.9, 4.5 Hz, 1H), 1.37 (d, J = 6.5 Hz, 6H), 1.28 (d, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 83.39, 63.88, 51.88, 28.15, 22.83, 15.24. **Compound 816:** ^{1}H NMR, (400 MHz, DMSO-d_6) δ 5.67 (d, J = 6.3 Hz, 1H), 3.36 (dd, J = 8.4, 6.2 Hz, 1H), 3.27 – 3.19 (m, 1H), 1.37 (d, J = 6.5 Hz, 3H), 1.31 (d, J = 3.9 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 88.45, 57.99, 48.86, 25.74, 21.63, 19.17.

[0585] **Compound 817:** Compound **815** (0.2 g, 1.2 mmol) was dissolved in anhydrous ethyl acetate (6 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.32 mL, 1.8 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.41 mL, 1.8 mmol) were added, and the mixture was stirred at room temperature for 3 hours. The reaction was

quenched, diluted with ethyl acetate (20 mL), washed with 5% NaCl (3 x 40 mL), saturated NaCl (1 x 40 mL), dried over Na_2SO_4, filtered, and concentrated. The product was isolated by silica gel flash chromatography on a standard 24 g silica column quenched with triethylamine (10 mL), using gradient of ethyl acetate in hexane (1:9 to 1:2). The product-containing fractions were concentrated, chased with acetonitrile (2x), and dried under high vacuum. **Compound 817** was obtained as a slowly crystallizing yellow oil, 54% yield (0.24 g). ^{1}H NMR (500 MHz, Acetonitrile-d_3) δ 4.19 – 4.10 (m, 1H), 4.01 – 3.64 (m, 5H), 2.75 – 2.63 (m, 2H), 1.52 (s, 3H), 1.50 – 1.37 (m, 6H), 1.27 – 1.20 (m, 12H). ^{31}P NMR (202 MHz, Acetonitrile-d_3) δ 152.39, 150.75.

Scheme 7



818 819 820 821

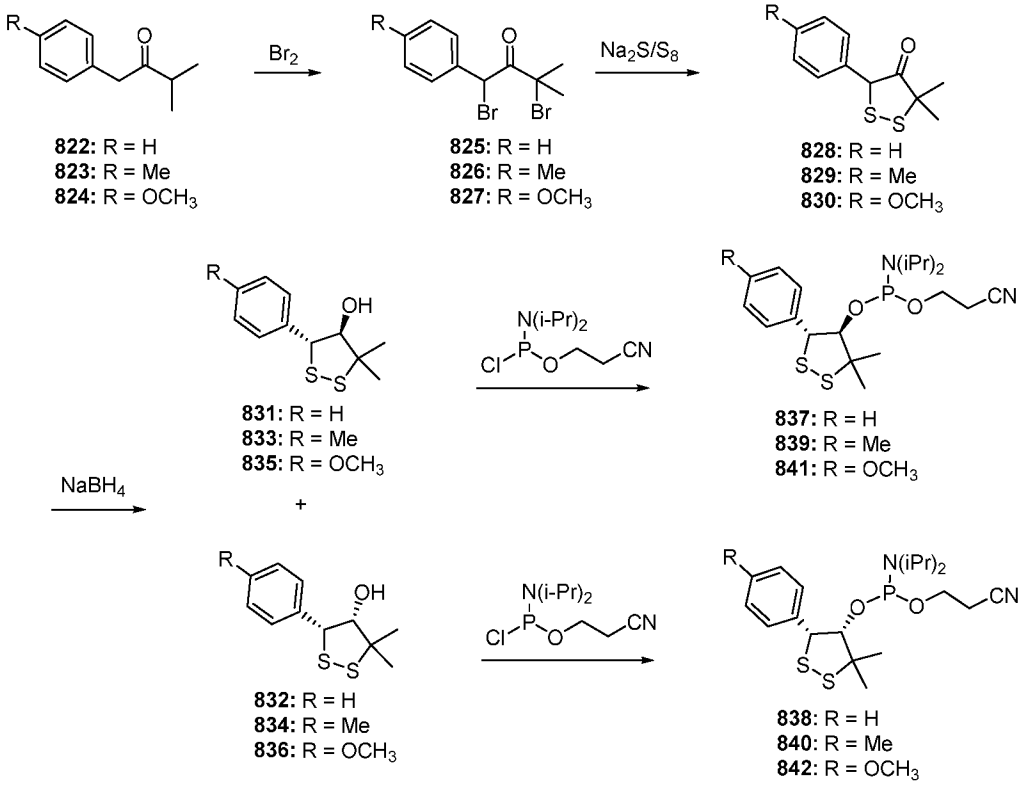
[0586] **Compound 819:** A suspension of sodium sulfide nonahydrate (4.08 g, 17 mmol) and elemental sulfur (1.09 g, 34 mmol) in MMA (N-methylacetamide) (35 mL) was stirred at 30 °C overnight to form homogeneous yellow solution. A solution of dibromoketone **818** (2.4 mL, 14 mmol) in MMA (10 mL) was added dropwise for ~ 20 minutes while maintaining bath temperature at 30 °C. The mixture was stirred at 30 °C for additional 3 hours, cooled to room temperature, and quenched by addition of 5% aqeous NaCl (200 mL). The mixture was extracted with ethyl acetate, the organic phase was separated, washed with 5% aqeous NaCl, saturated NaCl, and dried over anhydrous sodium sulfate. The solvent was evaporated in vacuum to afford crude residue (2.49 g) that was purified over a column of silica gel with gradient of 0 to 10% of ethyl acetate in hexane to afford 1.18 g (48%) of pure tetramethyl ketone **819**. ^{1}H NMR (400 MHz, CD3CN): δ 1.50 (s, 12H). ^{13}C NMR (126 MHz, CD3CN): δ 215.4; 58.6; 25.7.

[0587] **Compound 820:** Sodium borohydride (420 mg, 11 mmol) was added portion wise over period of 3 hours to a cooled (0 °C) and stirred solution of ketone **819** (0.78 g, 4.4 mmol) and acetic acid (0.5 mL, 8.7 mmol) in dry ethanol (15 mL) under Ar atmosphere. The mixture was stirred at 0 °C for additional 3 hours, the cooling bath was removed, and the mixture was quenched by addition of saturated ammonium chloride (30 mL) and ethyl acetate (10 mL). The mixture was allowed to warm up to room temperature, water (5 mL) was added to dissolve solids, and the mixture was stirred vigorously in the presence of air for 48

hours. The organic phase was separated, washed with saturated NaCl, and dried over anhydrous sodium sulfate. The solvent was removed in vacuum to afford crude product (0.84 g) that was purified over a column of silica gel with gradient 5 to 20% of ethyl acetate in hexane to afford 0.65 g (83%) of **820** as slowly crystallizing yellowish liquid. ^{1}H NMR (500 MHz, CD_3CN): δ 3.50 (d, J = 6.5 Hz, 1H); 3.43 (d, J = 7.0 Hz, 1H); 1.44 (s, 6H); 1.36 (s, 6H).

[0588] **Compound 821:** 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.45 mL, 2 mmol) was added to a cooled (0 °C) and stirred solution of tetramethyl carbinol **820** (0.27 g, 1.5 mmol) and N, N-diisopropylethylamine (0.35 mL, 2 mmol) in anhydrous ethyl acetate (7 mL) under Ar atmosphere. The cooling bath was removed, the mixture was stirred at room temperature for 24 hours, cooled to 0 °C and quenched by addition of saturated solution of sodium bicarbonate. The organic phase was separated, dried over anhyd. sodium sulfate, and the crude residue was purified over a column of silica gel with gradient of dichloromethane in hexane 35 to 100% to afford 0.37 g (65%) of pure amidite **821** as a yellowish oil. ^{1}H NMR (400 MHz, CD_3CN): δ 3.89-3.80 (m, 1H); 3.77 (d, J = 12.4 Hz, 1H); 3.73-3.59 (m, 3H); 2.65 (t, J = 6.0 Hz, 2H); 1.55 (s, 3H); 1.50 (s, 3H); 1.44 (s, 3H); 1.42 (s, 3H); 1.22 (d, J = 6.8 Hz, 6H); 1.18 (d, J = 6.8 Hz, 6H). ^{31}P NMR (202 MHz, CD_3CN): δ 150.8.

Scheme 8



[0589] **Compound 825:** 3-methyl-1-phenyl-2-butanone, Compound **822** (4.0 g, 24.7 mmol) was dissolved in anhydrous diethyl ether (20 mL) under argon atmosphere. To the ketone solution was added 3 drops of a solution of bromine (7.9g, 2.5 mL, 49.3 mmol) and DCM (10 mL) to initiate the reaction. Once the reaction changed from orange to colorless, the remaining bromine solution was added dropwise over a period of one hour. The reaction was stirred for additional 2 hours, then diluted with diethyl ether (100 mL), and charged to a stirring solution of 5% NaCl (100 mL) portion-wise. The organic layer was then washed with 5% NaCl (3 x 100 mL), 5% $Na_2S_2O_5$ (1 x 100 mL), and saturated NaCl (1 x 100 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The brownish crude residue was purified over a column of silica gel with gradient of 2% to 7% ethyl acetate in hexane to afford pure compound **825** as a white solid, 96% yield (7.6 g). ^{1}H NMR (400 MHz, DMSO) δ 7.67 – 7.63 (m, 2H), 7.41 – 7.33 (m, 3H), 6.60 (s, 1H), 1.99 (s, 3H), 1.79 (s, 3H).

[0590] **Compound 826:** 1-(4-methyl)-3-methylbutan-2-one, Compound **823** (4.51 g, 25.6 mmol) was dissolved in anhydrous diethyl ether (20 mL) under argon atmosphere. To the ketone solution was added 10 drops of a solution of bromine (8.18 g, 2.62 mL, 51.2 mmol) and DCM (15 mL) to initiate the reaction. Once the reaction changed from orange to lighter orange, the remaining bromine solution was added dropwise over a period of one hour. The reaction was stirred for additional 2 hours, then diluted with diethyl ether (100 mL), and charged to a stirring solution of 5% NaCl (100 mL) portion-wise. The organic layer was then washed with 5% NaCl (3 x 100 mL), 5% $Na_2S_2O_5$ (1 x 100 mL), and saturated NaCl (1 x 100 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The brownish crude residue was purified over a column of silica gel with gradient of 3% to 20% ethyl acetate in hexane to afford pure compound **826** as a white solid, 79% yield (6.77 g). ^{1}H NMR (500 MHz, DMSO) δ 7.53 (d, J = 8.2 Hz, 2H), 7.19 (d, J = 7.7 Hz, 2H), 6.57 (s, 1H), 2.28 (s, 3H), 1.97 (s, 3H), 1.78 (s, 3H).

[0591] **Compound 827:**1-(4-methoxyphenyl)-3-methylbutan-2-one, Compound **824** (4.82 g, 25.1 mmol) was dissolved in anhydrous diethyl ether (20 mL) under argon atmosphere. To the ketone solution was added 3 drops of a solution of bromine (8.01 g, 2.6 mL, 50.1 mmol) and DCM (15 mL) to initiate the reaction. Once the reaction changed from orange to lighter orange, the remaining bromine solution was added dropwise over a period of one hour. The reaction was stirred for additional 2 hours, then diluted with diethyl ether (100 mL), and charged to a stirring solution of 5% NaCl (100 mL) portion-wise. The organic layer was then washed with 5% NaCl (3 x 100 mL), 5% $Na_2S_2O_5$ (1 x 100 mL), and saturated

NaCl (1 x 100 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The brownish crude residue was purified over a column of silica gel with gradient of 0% to 15% ethyl acetate in hexane to afford pure compound **827** as a white solid, 91% yield (8.0 g). ^{1}H NMR (600 MHz, DMSO) δ 7.59 (d, J = 6.8 Hz, 2H), 6.95 (d, J = 6.8 Hz, 2H), 6.61 (s, 1H), 3.76 (s, 3H), 1.97 (s, 3H), 1.79 (s, 3H).

[0592] **Compound 828:** To a reactor containing N-methylacetamide (40 mL), heated to 33 °C, was charged sodium sulfide nonahydrate (6.0 g, 25 mmol) and sulfur (1.6 g, 50 mmol). This suspension was stirred overnight at 35 °C to dissolve the solids. The mixture was cooled to 30 °C, then Compound **825** (4.0 g, 25.0 mmol) was added. The reaction was stirred for 3 hours, then quenched by adding to a stirring solution of 5% NaCl (200 mL). The mixture was extracted with ethyl acetate (100 mL) and washed with 5% NaCl (2 x 150 mL) and saturated NaCl (1 x 150 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was diluted with hexane (100 mL), and the precipitated residual sulfur was removed by vacuum filtration. The filtrate was concentrated to afford a crude residue which was purified over a column of silica gel with gradient of 4% to 10% ethyl acetate in hexane to afford pure compound **828** as a yellow solid, 89% yield (2.49 g). ^{1}H NMR (500 MHz, DMSO) δ 7.44 – 7.25 (m, 6H), 5.28 (s, 1H), 1.62 (s, 3H), 1.52 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 210.06, 135.77, 129.16, 128.75, 128.32, 58.97, 56.66, 24.56, 24.16.

[0593] **Compound 829:** To a reactor containing N-methylacetamide (40 mL), heated to 33°C, was charged sodium sulfide nonahydrate (6.0 g, 25 mmol) and sulfur (1.6 g, 50 mmol). This suspension was stirred overnight at 35 °C to dissolve the solids. The mixture was cooled to 30 °C, then Compound **826** (4.0 g, 25.0 mmol) was added. The reaction was stirred for 3 hours, then quenched by adding to a stirring solution of 5% NaCl (200 mL). The mixture was extracted with ethyl acetate (100 mL) and washed with 5% NaCl (2 x 150 mL) and saturated NaCl (1 x 150 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was diluted with hexane (100 mL), and the precipitated residual sulfur was removed by vacuum filtration. The filtrate was concentrated to afford a crude residue which was purified over a column of silica gel with gradient of 4% to 10% ethyl acetate in hexane to afford pure compound **829** as a yellow solid, 53% yield (1.51 g). ^{1}H NMR (500 MHz, DMSO) δ 7.17 (s, 4H), 5.23 (s, 1H), 2.28 (s, 3H), 1.62 (s, 3H), 1.50 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 210.21, 137.81, 132.70, 129.32, 129.09, 58.91, 56.59, 24.65, 24.19, 20.71.

[0594] **Compound 830:** To a reactor containing N-methylacetamide (30 mL), heated to 33°C, was charged sodium sulfide nonahydrate (4.71 g, 19.6 mmol) and sulfur (1.26 g, 39.2 mmol). This suspension was stirred overnight at 35 °C to dissolve the solids. The mixture was cooled to 30 °C, then Compound **827** (3.43 g, 9.8 mmol) was added. The reaction was stirred for 3 hours, then quenched by adding to a stirring solution of 5% NaCl (150 mL). The mixture was extracted with ethyl acetate (100 mL) and washed with 5% NaCl (2 x 150 mL) and saturated NaCl (1 x 150 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude product was diluted with hexane (100 mL), and the precipitated residual sulfur was removed by vacuum filtration. The filtrate was concentrated to afford a crude residue which was purified over a column of silica gel with gradient of 0% to 15% ethyl acetate in hexane to afford pure compound **830** as a yellow solid, 78% yield (1.75 g). ^{1}H NMR (600 MHz, DMSO) δ 7.21 (d, J = 6.9 Hz, 2H), 6.94 (d, J = 6.9 Hz, 2H), 5.24 (s, 1H), 3.75 (s, 3H), 1.63 (s, 3H), 1.50 (s, 3H). ^{13}C NMR (151 MHz, DMSO) δ 210.87, 159.73, 131.03, 127.99, 114.71, 59.26, 56.98, 55.65, 25.23, 24.72.

[0595] **Compounds 831 and 832:** Compound **828** (2.32 g, 10.34 mmol) was dissolved in ethanol (25 mL) under argon in an oven dried flask, and was then cooled to -78 °C. Acetic acid (0.62 g, 0.60 mL, 10.34 mmol) was charged, followed by $NaBH_4$ (0.39 g, 10.34 mmol). The reaction was stirred at -78 °C for 10 minutes, at 0 °C for an hour, and then at room temperature overnight. The reaction was cooled to 0 °C, and an additional aliquot of $NaBH_4$ (0.10 g, 2.59 mmol) was added. The reaction was stirred at 0 °C for 5 hours, and then quenched by slow addition of saturated NH_4Cl (15 mL). The mixture was diluted with ethyl acetate (80 mL), saturated NH_4Cl (40 mL) and water (35 mL). The mixture was stirred for 18 hours at room temperature. The organic layer was washed with 1:1 saturated NH_4Cl:water (1 x 50 mL), saturated $NaHCO_3$ (1 x 50 mL), and saturated NaCl (1 x 50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude yellow residue was purified over a column of silica gel with gradient of 6% to 10% ethyl acetate in hexane to afford early eluting compound **832** (racemic mixture) as a yellow solid (1.45 g, 62% yield) and late eluting compound **831** (racemic mixture) as a yellow solid (0.13 g, 6% yield). Compound **831:** ^{1}H NMR (500 MHz, DMSO) δ 7.51 – 7.42 (m, 2H), 7.38 – 7.24 (m, 3H), 5.68 (d, J = 6.7 Hz, 1H), 4.29 (d, J = 8.7 Hz, 1H), 3.93 (dd, J = 8.6, 6.6 Hz, 1H), 1.46 (s, 3H), 1.38 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 140.17, 128.44, 128.15, 127.59, 89.28, 58.61, 57.01, 25.32, 21.12. Compound **832**: ^{1}H NMR (500 MHz, DMSO) δ 7.56 – 7.46 (m, 2H), 7.33 – 7.22 (m, 3H), 5.23 (d, J = 7.2 Hz, 1H), 5.00 (d, J = 3.8 Hz, 1H), 3.94 (dd, J = 6.9, 3.8

Hz, 1H), 1.52 (s, 3H), 1.44 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 136.49, 130.01, 127.66, 127.40, 83.58, 65.70, 61.53, 28.68, 23.27.

[0596] **Compounds 833 and 834:** Compound **829** (1.25 g, 5.24 mmol) was dissolved in ethanol (13 mL) under argon in an oven dried flask, and was then cooled to -78 °C. Acetic acid (0.31 g, 0.30 mL, 5.24 mmol) was charged, followed by $NaBH_4$ (0.20 g, 5.24 mmol). The reaction was stirred at -78 °C for 10 minutes, at 0 °C for an hour, and then at room temperature overnight. The reaction was cooled to 0°C, and an additional aliquot of $NaBH_4$ (0.05 g, 1.31 mmol) was added. The reaction was stirred at 0 °C for 5 hours, and then quenched by slow addition of saturated NH_4Cl (10 mL). The mixture was diluted with ethyl acetate (50 mL), saturated NH_4Cl (25 mL) and water (20 mL). The mixture was stirred for 18 hours at room temperature. The organic layer was washed with 1:1 saturated NH_4Cl:water (1 x 50 mL), saturated $NaHCO_3$ (1 x 50 mL), and saturated NaCl (1 x 50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude yellow residue was purified over a column of silica gel with gradient of 5% to 20% ethyl acetate in hexanes to afford early eluting compound **834** (racemic mixture) as a yellow solid (0.87 g, 69% yield) and late eluting compound **833** (racemic mixture) as a yellow solid (0.052 g, 4% yield). Compound **833**: ^{1}H NMR (600 MHz, DMSO) δ 7.37 – 7.32 (m, 2H), 7.16 (d, J = 7.6 Hz, 2H), 5.66 (d, J = 6.7 Hz, 1H), 4.26 (d, J = 8.6 Hz, 1H), 3.91 (dd, J = 8.8, 6.5 Hz, 1H), 2.29 (s, 3H), 1.45 (s, 3H), 1.38 (s, 3H). Compound **834**: ^{1}H NMR (600 MHz, DMSO) δ 7.44 – 7.36 (m, 2H), 7.13 – 7.08 (m, 2H), 5.24 – 5.16 (m, 1H), 4.97 (d, J = 3.1 Hz, 1H), 3.93 – 3.86 (m, 1H), 2.28 (s, 3H), 1.52 (s, 3H), 1.44 (s, 3H). ^{13}C NMR (151 MHz, DMSO) δ 137.11, 133.81, 130.38, 128.75, 84.03, 66.08, 61.83, 29.28, 23.84, 21.17.

[0597] **Compound 835 and 836:** Compound **830** (1.9 g, 7.5 mmol) was suspended in ethanol (20 mL) under argon in an oven dried flask, and then cooled to -78 °C. Acetic acid (0.45 g, 0.43 mL, 7.5 mmol) was charged, followed by $NaBH_4$ (0.28 g, 7.5 mmol). The reaction was stirred at -78 °C for 10 minutes, at 0 °C for an hour, and then at room temperature overnight. The reaction was cooled to 0 °C, and an additional aliquot of $NaBH_4$ (0.05 g, 1.31 mmol) was added. The reaction was stirred at 0 °C for 5 hours, then quenched by slow addition of saturated NH_4Cl (40 mL) and water (35 mL). The mixture was stirred for 48 hours. The organic layer was washed with 1:1 saturated NH_4Cl:water (1 x 50 mL), saturated $NaHCO_3$ (1 x 50 mL), and saturated NaCl (1 x 50 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. The crude yellow residue was purified over a column of silica gel with 3:48.5:48.5 diethyl ether:DCM:hexanes to afford early eluting

compound **836** (racemic mixture) as a yellow solid (1.0 g, 52% yield) and late eluting compound **835** (racemic mixture) as a yellow solid (0.07 g, 4% yield). Compound **835**: ^{1}H NMR (600 MHz, DMSO) δ 7.37 (d, *J* = 8.7 Hz, 2H), 6.91 (d, *J* = 8.7 Hz, 2H), 5.66 (d, *J* = 6.7 Hz, 1H), 4.27 (d, *J* = 8.8 Hz, 1H), 3.89 (dd, *J* = 8.8, 6.6 Hz, 1H), 3.74 (s, 3H), 1.43 (s, 3H), 1.38 (s, 3H). ^{13}C NMR (151 MHz, DMSO) δ 159.21, 131.67, 128.54, 113.60, 83.93, 66.04, 61.41, 55.54, 29.36, 23.88. Compound **836**: ^{1}H NMR (600 MHz, DMSO) δ 7.45 (d, *J* = 8.7 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 2H), 5.27 (d, *J* = 7.2 Hz, 1H), 4.97 (d, *J* = 3.7 Hz, 1H), 3.85 (d, *J* = 3.3 Hz, 1H), 3.73 (s, 3H), 1.51 (s, 3H), 1.43 (s, 3H). ^{13}C NMR (151 MHz, DMSO) δ 159.29, 132.02, 129.81, 114.37, 89.34, 58.74, 57.19, 55.62, 26.10, 21.89.

[0598] **Compound 837:** Compound **831** (0.1 g, 0.44 mmol) was dissolved in anhydrous ethyl acetate (1 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.15 mL, 0.88 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.15 mL, 0.66 mmol) were added and the mixture was stirred at room temperature for 18 hours. The reaction was quenched, diluted with ethyl acetate (20 mL), washed with 5% NaCl (3 x 20 mL) and saturated NaCl (1 x 40 mL), dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified over a column of silica gel with gradient of 5% to 30% ethyl acetate in hexanes to afford pure compound **837** as a yellow oil, 77% yield (0.15 g). ^{1}H NMR (600 MHz, CD_3CN) δ 7.55 – 7.50 (m, 2H), 7.41 – 7.30 (m, 3H), 4.57 – 4.43 (m, 2H), 3.68 – 3.49 (m, 3H), 3.27 – 3.13 (m, 1H), 2.560 – 2.56 (m, 1H), 2.26 – 2.20 (m, 1H), 1.62 – 1.56 (m, 6H), 1.16 (d, *J* = 6.8 Hz, 3H), 1.10 (d, *J* = 6.8 Hz, 3H), 1.05 (d, *J* = 6.8 Hz, 3H), 0.97 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (151 MHz, CD_3CN) δ 140.19, 139.67, 129.22, 129.17, 129.14, 129.07, 128.60, 128.46, 92.49, 92.40, 92.22, 92.15, 59.68, 59.65, 59.62, 59.20, 59.16, 58.38, 58.25, 58.19, 58.05, 43.55, 43.47, 43.40, 43.31, 26.16, 25.94, 25.91, 24.37, 24.32, 24.29, 24.26, 24.20, 24.14, 22.59, 22.00, 20.39, 20.34, 20.14, 20.09. ^{31}P NMR (243 MHz, CD_3CN) δ 150.08, 148.64.

[0599] **Compound 839:** Compound **833** (0.05 g, 0.21 mmol) was dissolved in anhydrous ethyl acetate (0.5 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.07 mL, 0.42 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.07 mL, 0.31 mmol) were added and the mixture was stirred at room temperature for 18 hours. The reaction was quenched, diluted with ethyl acetate (10 mL), washed with 5% NaCl (3 x 15 mL) and saturated NaCl (1 x 15 mL), dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified over a column of silica gel with gradient of 5% to 30% ethyl acetate in hexanes to afford pure compound **839** as a yellow oil, 46% yield (0.042 g). ^{1}H NMR (600 MHz,

CD_3CN) δ 7.42 – 7.37 (m, 2H), 7.24 – 7.14 (m, 2H), 4.54 – 4.40 (m, 2H), 3.68 – 3.48 (m, 3H), 3.25 – 3.11 (m, 1H), 2.59 – 2.57 (m, 1H), 2.34 (d, J = 12.2 Hz, 3H), 2.26 – 2.20 (m, 1H), 1.61 – 1.56 (m, 5H), 1.16 (d, J = 6.8 Hz, 3H), 1.10 (d, J = 6.8 Hz, 3H), 1.05 (d, J = 6.8 Hz, 3H), 0.97 (d, J = 6.8 Hz, 3H). ^{13}C NMR (151 MHz, CD_3CN) δ 138.49, 138.43, 136.85, 136.42, 129.70, 129.67, 129.09, 129.08, 92.42, 92.31, 92.12, 92.04, 59.74, 59.71, 59.43, 59.40, 59.09, 58.99, 58.44, 58.30, 58.17, 58.03, 43.59, 43.51, 43.41, 43.32, 26.31, 26.05, 26.02, 24.38, 24.33, 24.30, 24.29, 24.26, 24.15, 24.09, 22.71, 22.12, 20.71, 20.70, 20.39, 20.34, 20.08, 20.03. ^{31}P NMR (243 MHz, CD_3CN) δ 150.32, 148.64.

[0600] **Compound 841:** Compound **835** (0.042 g, 0.16 mmol) was dissolved in anhydrous ethyl acetate (0.5 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.04 mL, 0.25 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.05 mL, 0.25 mmol) were added, and the mixture was stirred at room temperature for 18 hours. The reaction was quenched, diluted with ethyl acetate (10 mL), washed with 5% NaCl (3 x 15 mL) and saturated NaCl (1 x 15 mL), dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified over a column of silica gel with gradient of 5% to 30% ethyl acetate in hexane to afford pure compound **841** as a yellow oil, 44% yield (0.033 g). ^1H NMR (600 MHz, CD_3CN) δ 7.46 – 7.40 (m, 2H), 6.97 – 6.87 (m, 2H), 4.52 – 4.37 (m, 2H), 3.80 (d, J = 11.7 Hz, 3H), 3.70 – 3.19 (m, 4H), 2.59 (t, J = 5.9 Hz, 1H), 2.31 – 2.27 (m, 1H), 1.63 – 1.55 (m, 6H), 1.16 (d, J = 6.8 Hz, 3H), 1.11 (d, J = 6.8 Hz, 3H), 1.06 (d, J = 6.8 Hz, 3H), 0.98 (d, J = 6.8 Hz, 3H).^{31}P NMR (243 MHz, CD_3CN) δ 150.16, 148.78.

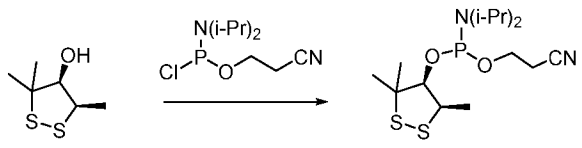
[0601] **Compound 838:** Compound **832** (0.40 g, 1.77 mmol) was dissolved in anhydrous ethyl acetate (9 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.4 mL, 2.65 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.59 mL, 2.65 mmol) were added and the mixture was stirred at room temperature for 18 hours. The reaction was quenched, diluted with ethyl acetate (40 mL), washed with 5% NaCl (3 x 80 mL) and saturated NaCl (1 x 80 mL), dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified over a column of silica gel with gradient of 5% to 30% ethyl acetate in hexane to afford pure compound **838** as a yellow oil, 80% yield (0.60 g). ^1H NMR (400 MHz, CD_3CN) δ 7.48 – 7.42 (m, 2H), 7.34 – 7.21 (m, 3H), 5.01 (d, J = 5.6 Hz, 1H), 4.36 – 4.31 (m, 1H), 3.76 – 3.66 (m, 1H), 3.62 – 3.43 (m, 3H), 2.64 – 2.55 (m, 2H), 1.66 (s, 3H), 1.61 (s, 3H), 1.04 (d, J = 6.8 Hz, 6H), 0.94 (d, J = 6.8 Hz, 6H). ^{31}P NMR (162 MHz, CD_3CN) δ 150.93.

[0602] **Compound 840:** Compound **834** (0.61 g, 2.53 mmol) was dissolved in anhydrous ethyl acetate (10 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.66 mL, 3.8

mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.85 mL, 3.8 mmol) were added and the mixture was stirred at room temperature for 18 hours. The reaction was quenched, diluted with ethyl acetate (50 mL), washed with 5% NaCl (3 x 50 mL) and saturated NaCl (1 x 50 mL), dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified over a column of silica gel with gradient of 5% to 30% ethyl acetate in hexane to afford pure compound **840** as a yellow oil, 83% yield (0.93 g). ^{1}H NMR (600 MHz, CD_3CN) δ 7.43 – 7.32 (m, 2H), 7.19 – 7.10 (m, 2H), 5.06 – 4.97 (m, 1H), 4.36 – 4.29 (m, 1H), 3.77 – 3.23 (m, 4H), 2.67 – 2.39 (m, 2H), 2.36 – 2.27 (m, 3H), 1.70 – 1.55 (m, 6H), 1.13 – 0.93 (m, 12H). ^{13}C NMR (151 MHz, CD_3CN) δ 138.08, 137.85, 134.25, 133.76, 131.16, 130.77, 129.34, 128.85, 119.28, 86.81, 86.73, 86.00, 85.94, 64.40, 62.96, 62.94, 61.09, 58.37, 58.28, 58.24, 58.13, 43.69, 43.60, 43.59, 43.51, 27.99, 27.56, 27.54, 24.59, 24.52, 24.47, 24.43, 24.39, 24.31, 24.27, 24.22, 23.83, 23.80, 20.73, 20.69, 20.56, 20.51, 20.41, 20.36. ^{31}P NMR (243 MHz, CD_3CN) δ 151.40, 149.14.

[0603] **Compound 842:** Compound **836** (0.50 g, 1.95 mmol) was dissolved in anhydrous ethyl acetate (8 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.51 mL, 2.9 mmol) and 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.65 mL, 2.9 mmol) were added and the mixture was stirred at room temperature for 18 hours. The reaction was quenched, diluted with ethyl acetate (50 mL), washed with 5% NaCl (3 x 50 mL) and saturated NaCl (1 x 50 mL), dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified over a column of silica gel with gradient of 5% to 30% ethyl acetate in hexane to afford pure compound **842** as a yellow oil, 77% yield (0.68 g). ^{1}H NMR (600 MHz, CD_3CN) δ 7.48 – 7.35 (m, 2H), 6.92 – 6.83 (m, 2H), 5.05 – 4.98 (m, 1H), 4.34 – 4.24 (m, 1H), 3.84 – 3.26 (m, 7H), 2.66 – 2.42 (m, 2H), 1.71 – 1.54 (m, 6H), 1.15 – 0.95 (m, 12H). ^{13}C NMR (151 MHz, CD_3CN) δ 159.94, 159.84, 132.42, 132.06, 131.44, 129.25, 128.73, 119.30, 114.13, 113.56, 86.68, 86.60, 85.84, 85.78, 64.31, 62.83, 62.79, 62.46, 62.44, 60.71, 58.39, 58.31, 58.25, 58.16, 55.49, 55.48, 43.71, 43.62, 43.57, 43.49, 27.95, 27.51, 27.49, 24.55, 24.51, 24.50, 24.44, 24.40, 24.38, 24.33, 24.33, 24.28, 23.77, 23.75, 22.94, 20.56, 20.52, 20.46, 20.40. ^{31}P NMR (243 MHz, CD_3CN) δ 151.42, 149.09.

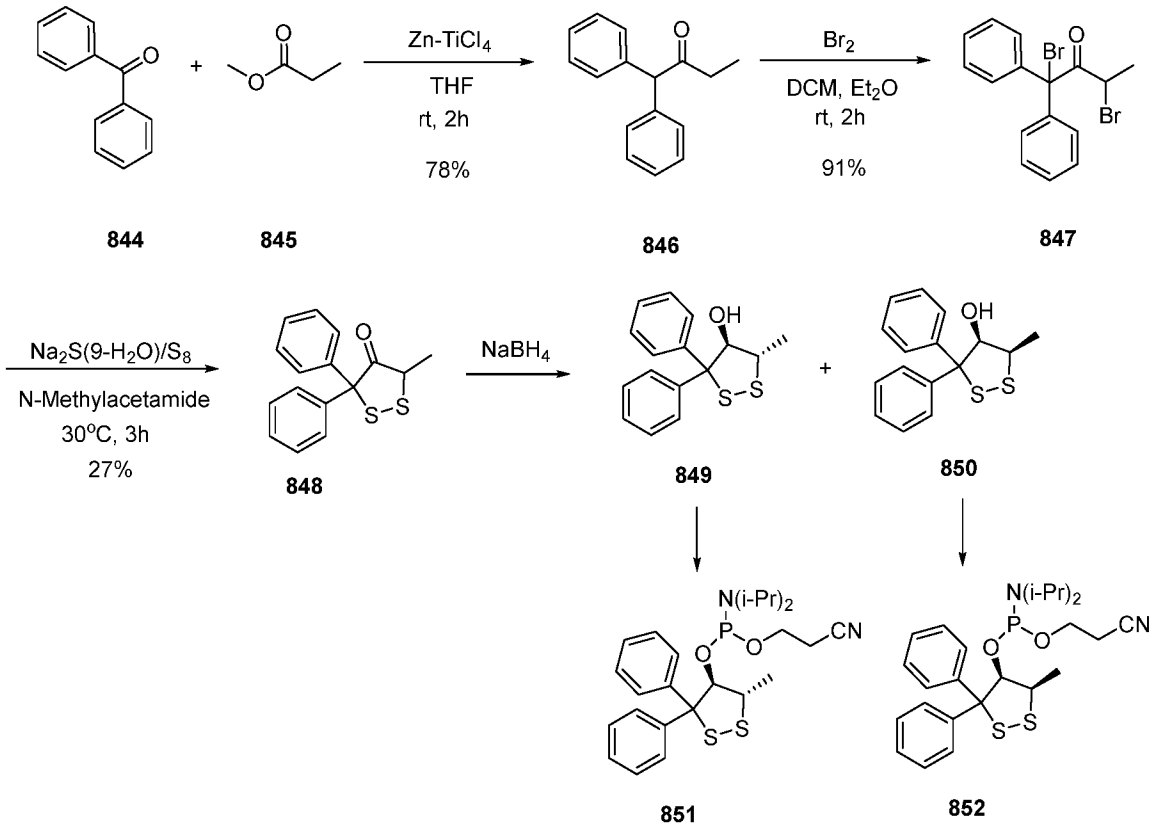
Scheme 9



816 843

[0604] **Compound 843:** Compound **816** (0.4 g, 2.4 mmol) was dissolved in anhydrous ethyl acetate (12 mL) under an inert atmosphere. *N,N*-Diisopropylethylamine (0.41 g, 3.2 mmol) was added followed by addition of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.75 g, 3.2 mmol), and the mixture was stirred at room temperature for 3 hours. The reaction was quenched with a solution of saturated sodium bicarbonate and ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to give a crude residue which was purified by silica gel flash chromatography to afford pure Compound **843** as a yellow oil, 89% yield (0.79 g). ^{1}H NMR (400 MHz, CD_3CN) δ 3.91 – 3.42 (m, 6H), 2.69 – 2.61 (m, 2H), 1.52 – 1.43 (m, 9H), 1.23 – 1.15 (m, 12H). ^{13}C NMR (101 MHz, CD_3CN) δ 119.62, 92.89, 92.76, 92.58, 92.46, 60.15, 60.10, 59.76, 59.74, 59.11, 59.07, 58.91, 58.87, 52.19, 52.15, 51.96, 44.17, 44.15, 44.05, 44.02, 26.90, 26.68, 26.63, 25.10, 25.02, 24.96, 24.94, 24.88, 23.39, 22.91, 21.04, 20.96, 19.92, 19.81, 19.75. ^{31}P NMR (162 MHz, CD_3CN) δ 150.00, 149.81.

Scheme 10



[0605] **Ketone 846**: To a 1L three-neck flask equipped with a reflux condenser were added methyl propionate **845** (6.48 g, 73.5 mmol), diphenylmethanone **844** (6.70 g, 36.8 mmol) and zinc powder (9.62 g, 147 mmol) under argon atmosphere. Anhydrous THF

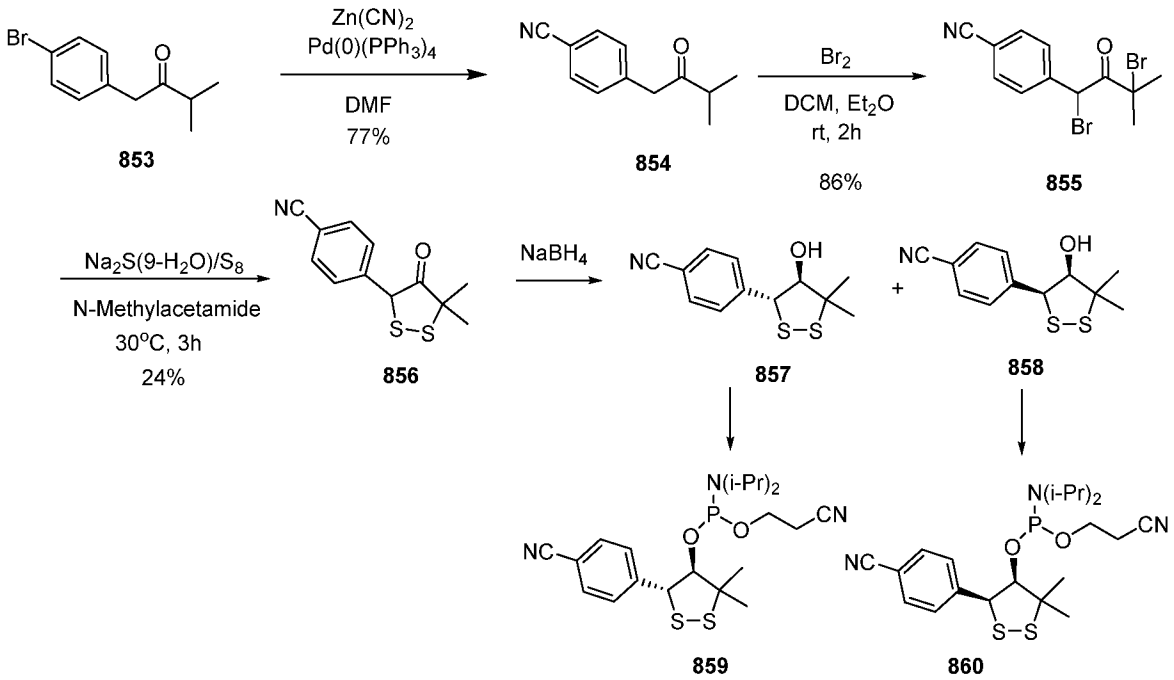
(180 mL) was added to the mixture with stirring. The suspension was cooled to 0-5°C in ice-water bath, and titanium (IV) chloride (13.95 g, 8.1 mL, 73.5 mmol) was slowly added to the mixture. The dark-blue suspension was stirred for 2 hours at 25 °C followed by heating at 50°C for 6 hours. The mixture was cooled to room temperature, and 1M HCl (800 mL) was added. The mixture was stirred at room temperature for 10 minutes and extracted with ethyl acetate (250 mL x 3). The organic layers were combined, washed with aqueous NaCl, and dried over $MgSO_4$. After the solid was filtered and solvent was removed in vacuum, the residue was purified by flash column chromatography on silica gel (330g, 120 mL/min, gradient of 30% DCM to 60% of DCM in hexanes) to give compound **846**: (6.44 g, 78 %). ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.93 (t, 3H, J = 6Hz), 2.56 (q, 2H, J = 6Hz),5.39(s,1H), 7.23-7.27(m, 6H), 7.31-7.34 (m, 4H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 8.5, 35.8, 62.8, 127.3, 129.0, 129.3, 139.6, 209.4.

[0606] **Dibromo-ketone 847**: 1,1-Diphenyl-butan-2-one (**846**) (7.2 g, 32.1 mmol) was dissolved in anhydrous diethyl ether (45 mL) under argon atmosphere. 12 drops of a solution of bromine (12.8 g, 80.3 mmol) in anhydrous DCM (15 mL) were added to initiate reaction. Once the reaction mixture solution changed color from orange to almost colorless, the remaining bromine solution was added dropwise over a period of 35 minutes. The reaction mixture was stirred for an additional 2 hours, diluted with diethyl ether (120 mL), and slowly, portion-wise poured to a stirring solution of 5% NaCl (150 mL). The organic layer was separated, washed with 5% NaCl (2 x 150 mL), 5% sodium meta-bisulfite (1 x 150 mL), and saturated NaCl (1 x 150 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under vacuum to give yellowish liquid which slowly solidified upon cooling to give compound **847**: 95% purity, 11.2 g (91%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.69 (d, 3H, J= 5Hz), 4.99 (q, 2H, J=5Hz), 7.30-7.32(m, 4H), 7.41-7.48 (m, 6H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 24.5, 44.4, 76.1, 128.9, 129.1, 129.5, 129.8, 130.0, 130.1, 137.6, 138.5, 198.7.

[0607] **Cyclic ketone 848**: To a 100 mL RBF containing N-methylacetamide (15 mL) and heated to 33°C was added sodium sulfide nonahydrate (1.20 g, 5.0 mmol) and sulfur (320 mg, 10 mmol). The suspension was stirred for 24 hours at 35 °C to dissolve the solids. The reaction mixture was cooled to 30 °C, and a solution of **847** (1.27 g, 3.33 mmol) in N-methylacetamide (3 mL) was added slowly, dropwise to the reaction mixture. The reaction was stirred at 30 °C for 3 hours, and quenched by pouring to a stirred solution of 5% NaCl (60 mL). The mixture was extracted with ethyl acetate (50 mL), washed with 5% NaCl (3 x

40 mL) and saturated NaCl (1 x 40 mL). The organic layer was separated and dried over Na_2SO_4, filtered, and concentrated under vacuum to a yellow oil which was triturated with hexanes (80 mL) to precipitate solid sulfur that was removed by filtration. The resulted filtrate was concentrated under vacuum to give yellow oil which was purified by flash column chromatography on silica gel (40 g, 20 mL/min, elution with gradient of 5% to 35% of ethyl acetate in hexanes). The product-containing fractions were combined and concentrated under vacuum to afford compound **848** as a yellow oil: 265 mg (27%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.08 (d, 3H, *J*= 5Hz), 4.33 (q, 1H, *J*=5Hz), 7.30-7.39 (m, 8H), 7.49-7.51 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 11.3, 51.6, 68.24, 125.9, 126.1, 126.3, 126.4, 126.8, 126.9, 137.7, 140.9, 205.5.

Scheme 11



[0608] **Ketone 854**: To an oven-dried 100 mL round bottom flask were added 1-(4-bromophenyl)-3-methyl-butan-2-one (**853**) (3.50 g, 14.5 mmol), palladium (0) tetrakis-triphenylphosphine (1.34 g, 1.2 mmol) and zinc cyanide (1.70 g, 14.5 mmol). Anhydrous DMF (35 mL) was added, the reaction mixture was then degassed and heated at 90 °C under argon atmosphere overnight. The mixture was cooled, diluted with 150 mL of EtOAc and washed with ammonium hydroxide (2M, 150 mL x 2), followed by saturated $NaHCO_3$ (140 mL) and saturated NaCl (100 mL). The organic layer was separated, dried over sodium sulfate, filtered, and concentrated under vacuum to give 3.22 g of crude residue. The crude

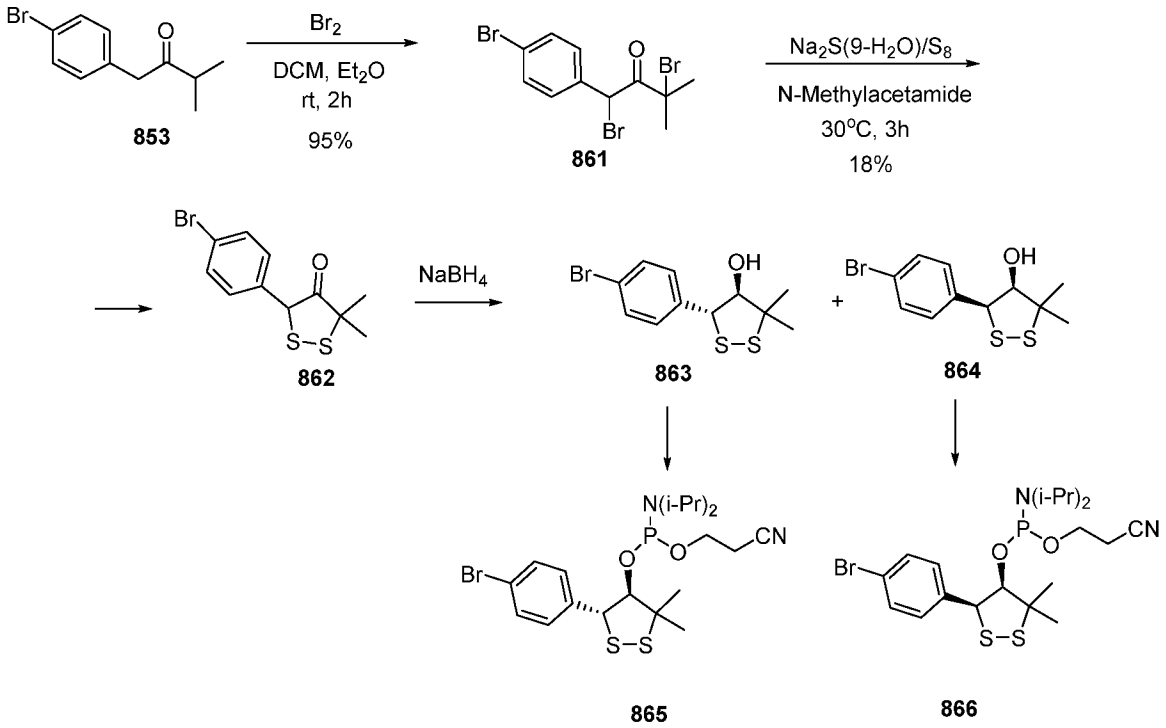
residue was purified by flash column chromatography on silica gel (220 g, 60 mL/min, gradient of 20% to 35% of ethyl acetate in hexanes) to afford colorless oil which slowly solidified to furnish compound **854** as a white solid: (2.13 g, 77%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.17 (d, 6H, *J*= 5Hz), 2.74 (m, 1H), 3.84 (s, 2H), 7.31-7.33(m, 2H), 7.62-7.64 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 18.2, 41.0, 47.1, 110.9, 118.8, 130.4, 132.3, 139.8, 210.2.

[0609] **Dibromo-ketone 855**: 1-(4-cyanophenyl)-3-methyl-butan-2-one **854** (2.10 g, 11.2 mmol) was dissolved in anhydrous diethyl ether (12 mL) under argon atmosphere. 12 Drops of a solution of bromine (3.85 g, 24.1 mmol) in anhydrous DCM (5 mL) was added to initiate the reaction. Once the reaction mixture solution changed color from orange to almost colorless, the remaining bromine solution was added dropwise over a period of 25 minutes. The reaction was then stirred for an additional 2 hours, diluted with diethyl ether (40 mL) and slowly, portion wise poured to a stirring solution of 5% NaCl (55 mL). The organic layer was separated, washed with 5% NaCl (55 mL x 2), 5% sodium meta-bisulfite (1x 55 mL), and saturated NaCl (1 x 55 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to give compound **855** (~95% purity) as a yellow liquid which slowly solidified upon cooling: 3.35 g (86%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.79 (s, 3H), 2.05 (s, 3H), 6.04 (s, 1H), 7.59 (d, 2H, *J*= 8Hz), 7.73 (d, 2H, *J*=8Hz). ^{13}C NMR (126 MHz, $CDCl_3$) δ 27.2, 28.5, 41.5, 62.2, 111.1, 116.3, 128.2, 130.5, 138.9, 194.1.

[0610] **Cyclic ketone 856**: To a 100 mL RBF containing N-methylacetamide (15 mL) and heated to 33 °C was added sodium sulfide nonahydrate (1.20 g, 5.0 mmol) and sulfur (320 mg, 10 mmol). The suspension was stirred for 24 hours at 35 °C to dissolve solids. The reaction mixture was cooled to 30 °C, and a solution of **855** (1.27 g, 3.33 mmol) in N-methylacetamide (3 mL) was added slowly, dropwise to the reaction mixture. The reaction was stirred at 30 °C for 3 hours and quenched by pouring to a stirred solution of 5% NaCl (60 mL). The mixture was extracted with ethyl acetate (50 mL), washed with 5% NaCl (3 x 40 mL) and saturated NaCl (1 x 40 mL). The organic layer was separated and dried over Na_2SO_4, filtered, and concentrated under vacuum to a yellow oil which was triturated with hexanes (80 mL) to precipitate solid sulfur that was removed by filtration. The resulted filtrate was concentrated under vacuum to give yellow oil which was purified by flash column chromatography on silica gel (40 g, 20 mL/min, elution with gradient of 5% to 35% of ethyl acetate in hexanes). The product-containing fractions were combined and concentrated under vacuum to afford **856** as a yellow oil: 265 mg (27%). ^{1}H NMR (600

MHz, DMSO-d_6) δ 1.56 (s, 3H), 1.60 (s, 3H), 5.50 (s, 1H), 7.52-7.54(m, 2H), 7.86-7.88 (m, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 24.5, 24.8, 57.5, 58.3, 111.6, 119.0, 130.5, 133.2, 142.0, 209.7.

Scheme 12

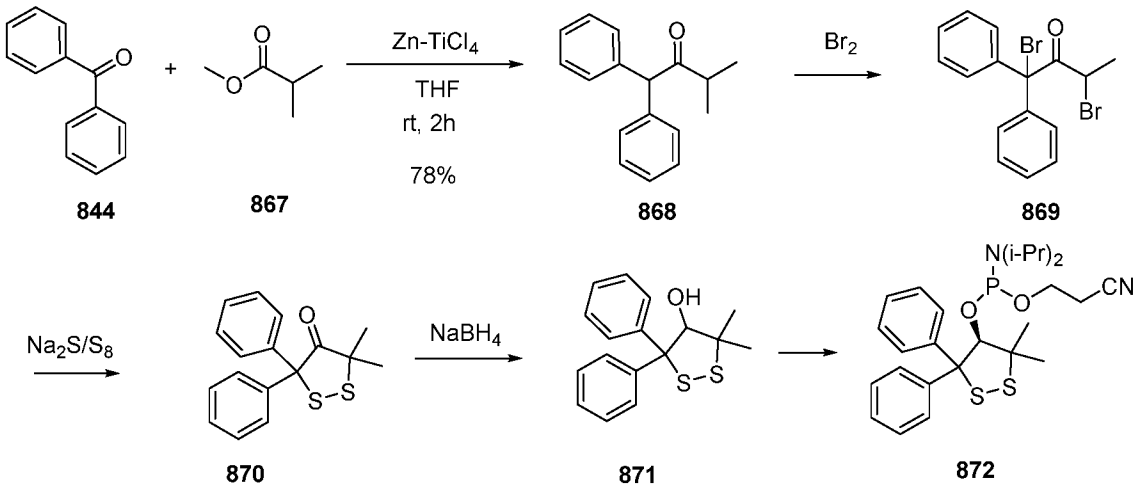


[0611] **Dibromo-ketone 861**: 1-(4-bromophenyl)-3-methyl-butan-2-one **853** (2.00 g, 8.3 mmol) was dissolved in anhydrous diethyl ether (12 mL) under argon atmosphere. 12 Drops of a solution of bromine (3.98 g, 24.9 mmol) in anhydrous DCM (6 mL) were added to initiate reaction. Once the reaction mixture changed color from orange to almost colorless, the remaining bromine solution was added dropwise over a period of 25 minutes. The mixture was stirred for an additional 2 hours, diluted with diethyl ether (40 mL), and slowly, portion-wise poured to a stirring solution of 5% NaCl (60 mL). The organic layer was separated, washed with 5% NaCl (60 mL x 2), 5% sodium meta-bisulfite (60 mL x 1) and saturated NaCl (60 mL x 1), dried over Na_2SO_4, filtered, and concentrated under vacuum to give compound **861** (~95% purity) as a yellow liquid which slowly solidified upon cooling: 3.15 g (95%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.74 (s, 3H), 1.98 (s, 3H), 6.00 (s, 1H), 7.17-7.45 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 29.3, 30.5, 44.5, 63.8, 123.6, 130.9, 132.0, 134.9, 196.6.

[0612] **Cyclic ketone 862**: To a 100 mL round bottom flask containing N-

methylacetamide (14 mL) and heated to 33 °C was added sodium sulfide nonahydrate (1.00 g, 4.2 mmol) and sulfur (0.268 g, 8.4 mmol). The suspension was stirred for 24 hours at 35 °C to dissolve the solids. The mixture was cooled to 30 °C, and a solution of compound **861** (1.11 g, 2.8 mmol) in N-methylacetamide (3 mL) was added slowly dropwise. The reaction mixture was stirred for 3 hours at 30 °C and quenched by pouring to a stirring solution of 5% NaCl (50 mL). The mixture was extracted with ethyl acetate (50 mL), and the organic layer was separated, washed with 5% NaCl (3 x 40 mL) and saturated NaCl (1 x 40 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under vacuum to a yellow oil which was triturated with hexanes (80 mL) to precipitate sulfur that was removed by filtration. The filtrate was concentrated under vacuum to afford a crude residue as a yellow oil which was purified by flash column chromatography on silica gel (40 g, 20 mL/min, elution with gradient of 5% to 35% of ethyl acetate in hexanes). The product-containing fractions were combined and concentrated under vacuum to afford compound **862** (~80% purity) as a yellow oil: (156 mg, 18%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.60 (s, 3H), 1.69 (s, 3H), 4.75 (s, 1H), 7.19-7.20 (m, 2H), 7.51-7.55 (m, 2H).

Scheme 13



[0613] **Ketone 868**: To a 1L three-neck flask equipped with a reflux condenser were added methyl 2-methylpropionic ester compound **867** (10.2 g, 100 mmol), diphenylmethanone compound **844** (9.10 g, 49.9 mmol) and zinc powder (13.06 g, 199.8 mmol) under argon atmosphere. Anhydrous THF (200 mL) was added with stirring, the suspension was cooled to 0-5 °C in ice-water bath, and titanium (IV) chloride (18.9 g, 10.9 mL, 100 mmol) was slowly added. The dark-blue suspension was stirred for 2 hours at 25 °C and then heated at 50 °C overnight. The reaction mixture was cooled to room temperature

and 1M HCl (800 mL) was added. The mixture was stirred at room temperature for 10 minutes, and extracted with ethyl acetate (300 mL x 3). The organic layers were combined, washed with aqueous NaCl, and dried over $MgSO_4$. After the solid was filtered and the solvent was removed in vacuum, the crude residue was purified by flash column chromatography on silica gel (330 g, 120 mL/min, gradient 30% to 60% of DCM in hexanes) to give compound **868**: (8.87 g, 74%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.15 (d, 6H, *J*= 6Hz), 2.83 (m, 1H), 5.33(s, 1H), 7.25-7.29(m, 6H), 7.32-7.35 (m, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 18.6, 41.0, 62.2, 127.1, 128.7, 129.0, 138.6, 212.1.

Example 2. Synthesis of oligonucleotides containing modified phosphate prodrug at the 5' end of the oligonucleotide

[0614] All oligonucleotides were synthesized as described here, or as otherwise described in Table 7. Oligonucleotides were synthesized at 1 or 10μmol scale using standard solid-phase oligonucleotide protocols, with 500-Å controlled pore glass (CPG) solid supports from Prime Synthesis and commercially available amidites from ChemGenes. The phosphoramidite solutions were 0.15 M in anhydrous acetonitrile with 15% THF as a co-solvent for 2'-O-methyl uridine, 2'-O-methyl cytidine, and the modified phosphate prodrugs. The modified phosphate prodrug monomers were coupled either on synthesizer or manually. For manual coupling, activator (0.25M 5-ethylthio-1H-tetrazole (ETT) in anhydrous ACN) was added followed by equal volume of prodrug solution. Solution was mixed for 20 minutes. Following coupling, the column was put on an ABI for oxidation or sulfurization. Oxidation (0.02M iodine in THF/pyridine/water) or sulfurization solution (0.1M 3-(dimethylaminomethylidene)amino-3H-1,2,4-dithiazole-3-thione (DDTT) in pyridine) was delivered to column for one minute or 30 seconds, respectively, and then held in solution for 10 minutes. This process was repeated for sulfurization. After completion of the solid-phase syntheses (SPS), the CPG solid support was washed with anhydrous acetonitrile and dried with argon. Oligonucleotides were deprotected by incubation with 5% diethylanolamine (DEA) in ammonia at room temperature for 2 hours.

[0615] Crude oligonucleotides were purified using strong anion exchange through a TSKgel SuperQ-5PW(20) resin with phosphate buffers (pH=8.5) containing sodium bromide at 65 °C. The appropriate fractions were pooled and desalted via SEC.

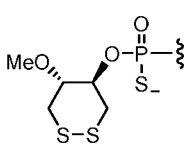
Table 7. Single oligonucleotide strands having modified phosphate prodrug at 5' end

Oligo ID	Sequence	Synthesizer /scale	Deprotection	Other
A-515432.1	(Pmd)dTdTdTdTdTdTdTdTdT	ABI394/ 1µmol	5% DEA in ammonia, 2h, RT	
A-515433.1	(Pmds)dTdTdTdTdTdTdTdTdT	ABI394/ 1µmol	5% DEA in ammonia, 2h, RT	
A-515488.2	(Pmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	Mermade192/ 1µmol	5% DEA in ammonia, 24h, 30°C	
A-515489.1	(Pmd)uCfacuUfuAfUfugagUfuUfcugugscsc	Mermade192/ 1µmol	5% DEA in ammonia, 24h, 30°C	
A-515490.1	(Pmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	Mermade192/1µmol	5% DEA in ammonia, 24h, 30°C	
A-515490.3	(Pmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	Mermade12/ 25µmol	5% DEA in ammonia, 24h, 30°C	
A-515491.1	(Pmds)uCfacuUfuAfUfugagUfuUfcugugscsc	Mermade192/ 1µmol	5% DEA in ammonia, 24h, 30°C	
A-515491.3	(Pmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	Mermade12/ 25µmol	5% DEA in ammonia, 24h, 30°C	
A-784093.1	(Pmds)uUfauaGfagcaagaAfcAfcuguususu	Mermade12/ 10µmol	5% DEA in ammonia, 24h, 30°C	
A-780495.1	(Pmds)uUfuagAfgUfGfaggaUfuAfaaaugsasg	Mermade12/ 10µmol	5% DEA in ammonia, 24h, 30°C	
A-1875172.1	(Cymd)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h, RT	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF Purification at 50°C
A-1875173.1	(Cymds)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h, RT	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF Purification at 50°C
A-1875174.1	(Pd)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h at RT, 5h at 65°C	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF Purification at 50°C
A-1875175.1	(Pds)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual	5% DEA in ammonia, 2h at RT, 5h at 65°C	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5%

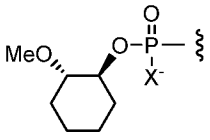
		coupling of prodrug		phenoxyacetic anhydride in THF Purification at 50°C
A-1875176.1	(Pdmd1)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h, RT	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF Unstable amidite, unsuccessful coupling
A-1875177.1	(Pdmd1s)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h, RT	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF Unstable amidite, unsuccessful coupling
A-1875178.1	(Pmmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h, RT	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF Purification at 50°C
A-1875179.1	(Pmmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h, RT	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF
A-1875180.1	(Ptmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL, Manual coupling of prodrug	5% DEA in ammonia, 2h, RT	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G Cap A: 5% phenoxyacetic anhydride in THF 50% n-1 Purification at 50°C
A-1875181.1	(Ptmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on Akta Oligopilot/ 12mL,	5% DEA in ammonia, 1h at 65°C, 16h at 30°C	Labile amidites (Pac protected) for 2'-OMeA, 2'-OMe G, 2'-F A, 2'-F G

		Manual coupling of prodrug		Cap A: 5% phenoxyacetic anhydride in THF 50% n-1 Purification at 50°C
A-2058840.1	(Cymds)uCfacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on ABI394 /10µmol, Manual coupling of prodrug	5% DEA in ammonia, 1h at 65°C, 16h at 30°C	
A-2058841.1	(Pds)uCfacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on ABI394/ 10µmol, Manual coupling of prodrug	5% DEA in ammonia, 10h at 65°C, 30h at 30°C	
A-2058842.1	(Pmmds)uCfacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on ABI394 /10µmol, Manual coupling of prodrug	5% DEA in ammonia, 1h at 65°C, 16h at 30°C	
A-2058843.1	(Ptmds)uCfacuUfuAfUfugagUfuUfcugugscsc	Precursor (up to prodrug) on ABI394/ 10µmol, Manual coupling of prodrug	5% DEA in ammonia, 1h at 65°C, 16h at 30°C	Double coupling

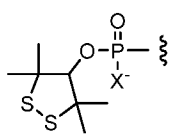
Upper case letter followed with f – 2'-deoxy-2'-fluoro (2'-F) sugar modification; lower case letter – 2'-*O*-methyl (2'-OMe) sugar modification; s – phosphorothioate (PS) linkage; VP – vinyl phosphonate; the prodrugs –



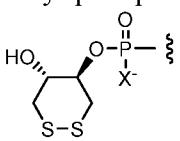
(Pmds) ,



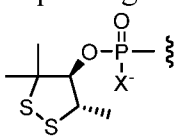
(Cymd) / (Cymds),



(Ptmd) / (Ptmds) ,



(Pd) / (Pds) , and



(Pmmd) / (Pmmds), wherein X is O or S.

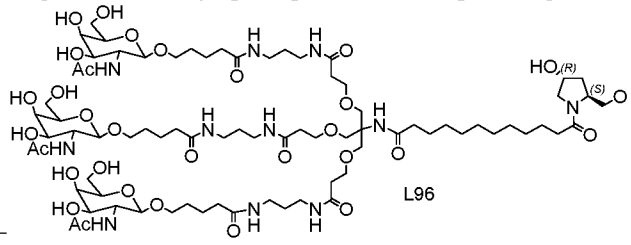
[0616] The siRNA duplexes having cyclic disulfide phosphate modifications at 5'-end of the antisense strand were synthesized and listed in Table 8.

Table 8: siRNAs having modified phosphate prodrug at the 5'-end of the antisense strand

Duplex ID	Oligo ID	Strand	Target	OligoSeq	Molecular Weight	Molecular Weight Found
AD-73603	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.12
	A-147465	antis	F12	usCfsacuUfuAfUfugagUfuUfcugugscsc	7514.87	7511.02
AD-291896	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.12
	A-447595	antis	F12	VPuCfacuUfuAfUfugagUfuUfcugugscsc	7558.74	7555.10
AD-326760	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-515491	antis	F12	(Pmds)uCfacuUfuAfUfugagUfuUfcugugscsc	7728.03	7723.02
AD-1023150	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875172	antis	F12	(Cymd)usCfsacuUfuAfUfugagUfuUfcugugscsc	7707.02	7703.08
AD-1023155	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875173	antis	F12	(Cymds)usCfsacuUfuAfUfugagUfuUfcugugscsc	7723.08	7719.03
AD-1023151	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875174	antis	F12	(Pd)usCfsacuUfuAfUfugagUfuUfcugugscsc	7729.06	7724.97
AD-1023156	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875175	antis	F12	(Pds)usCfsacuUfuAfUfugagUfuUfcugugscsc	7745.12	7740.95
AD-1023154	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875178	antis	F12	(Pmmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	7741.11	7737.01
AD-1023152	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875179	antis	F12	(Pmmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	7757.18	7752.99
AD-1023153	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875180	antis	F12	(Ptmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	7755.14	7751.03
AD-1023157	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-1875181	antis	F12	(Ptmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	7771.20	7767.01
AD-1144829	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-2058840	antis	F12	(Cymds)uCfacuUfuAfUfugagUfuUfcugugscsc	7690.95	7687.10

AD-1144831	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-2058841	antis	F12	(Pds)uCfacuUfuAfUfugagUfuUfcugugscsc	7712.99	7709.00
AD-1144832	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-2058842	antis	F12	(Pmmds)uCfacuUfuAfUfugagUfuUfcugugscsc	7725.05	7721.04
AD-1144830	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.65	8797.13
	A-2058843	antis	F12	(Ptmds)uCfacuUfuAfUfugagUfuUfcugugscsc	7739.07	7735.05

Upper case letter followed with f – 2'-F sugar modification; lower case letter – 2'-OMe sugar modification; s – phosphorothioate (PS) linkage; VP – vinyl phosphonate; the prodrugs are the same as in Table 7 above; the ligand —



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Example 3. ***In vitro*** **evaluation of siRNA duplexes containing modified phosphate prodrugs at the 5' end**

[0617] Transfection procedure: siRNA duplexes containing the modified phosphate prodrugs at the 5' end (Table 9) were transfected in primary mouse hepatocytes with RNAiMAX at 0.1, 1, 10, and 100 nm concentrations and analyzed 24 hours post-transfection. Percent F12 message remaining was determined by qPCR. The results were plotted against the control, as shown in **Figure 1**.

[0618] Free uptake procedure: siRNA duplexes containing the modified phosphate prodrugs at the 5' end (Table 9) were incubated with primary mouse hepatocytes at 0.1, 1, 10, and 100 nm concentrations in cell culture medium and analyzed after 48 hours. P ercentage of F12 message remaining was determined by qPCR. The results were plotted against the control, as shown in **Figure 2**.

Table 9. siRNA duplexes used for *in vitro* evaluation

Duplex ID	Sense Strand Sequence (5'-3')	Antisense Strand Sequence (5'-3')
AD-1023150	csascagaAfaCfUfCfaauaaagugaL96	(Cymd)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023151	csascagaAfaCfUfCfaauaaagugaL96	(Pd)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-	csascagaAfaCfUfCfaauaaagugaL96	(Pmmds)usCfsacuUfuAfUfugagUfu

1023152		Ufcugugscsc
AD-1023153	csascagaAfaCfUfCfaauaaagugaL96	(Ptmd)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023154	csascagaAfaCfUfCfaauaaagugaL96	(Pmmd)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023155	csascagaAfaCfUfCfaauaaagugaL96	(Cymds)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023156	csascagaAfaCfUfCfaauaaagugaL96	(Pds)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023157	csascagaAfaCfUfCfaauaaagugaL96	(Ptmds)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1144829	csascagaAfaCfUfCfaauaaagugaL96	(Cymds)uCfacuUfuAfUfugagUfuUfcugugscsc
AD-1144830	csascagaAfaCfUfCfaauaaagugaL96	(Ptmds)uCfacuUfuAfUfugagUfuUfcugugscsc
AD-1144831	csascagaAfaCfUfCfaauaaagugaL96	(Pds)uCfacuUfuAfUfugagUfuUfcugugscsc
AD-1144832	csascagaAfaCfUfCfaauaaagugaL96	(Pmmds)uCfacuUfuAfUfugagUfuUfcugugscsc
AD-326760	csascagaAfaCfUfCfaauaaagugaL96	(Pmds)uCfacuUfuAfUfugagUfuUfcugugscsc
AD-73603	csascagaAfaCfUfCfaauaaagugaL96	usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-291896	csascagaAfaCfUfCfaauaaagugaL96	VPuCfacuUfuAfUfugagUfuUfcugugscsc

Upper case letter followed with f – 2'-F sugar modification; lower case letter – 2'-OMe sugar modification; s – phosphorothioate (PS) linkage; VP – vinyl phosphonate; the prodrugs and ligands are the same as in Table 8 above.

[0619] Transfection procedure: siRNA duplexes containing the modified phosphate prodrugs at the 5' end were transfected in primary mouse hepatocytes with RNAiMAX at 0.1, 1, and 10 nm concentrations and analyzed 24 hours post-transfection. Percent F12 message remaining was determined by qPCR. The results were plotted against the control, as shown in **Figure 3**.

Table 10. siRNA duplexes used for *in vitro* evaluation in Figure 3

Duplex ID	Oligo ID	Strand	Target	Oligo Seq	Molecular Weight	Molecular Weight found
AD-73603	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-147465	antis	F12	usCfsacuUfuAfUfugagUfuUfcugugscsc	7514.875	7511.025
AD-291896	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128

	A-447595	antis	F12	VPuCfacuUfuAfUfugagUfuUfcugugscsc	7558.743	7555.05
AD-326758	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-515487	antis	F12	uCfacuUfuAfUfugagUfuUfcugugscsc	7482.744	7479.071
AD-326756	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-515488	antis	F12	(Pmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	7744.095	7738.993
AD-326757	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-515489	antis	F12	(Pmd)uCfacuUfuAfUfugagUfuUfcugugscsc	7711.964	7707.039
AD-326759	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-515490	antis	F12	(Pmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	7760.161	7754.97
AD-326760	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-515491	antis	F12	(Pmds)uCfacuUfuAfUfugagUfuUfcugugscsc	7728.03	7723.016
AD-454968	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-815956	antis	F12	PSusCfsacuUfuAfUfugagUfuUfcugugscsc	7611.928	7606.969
AD-454967	A-147464	sense	F12	csascagaAfaCfUfCfaauaaagugaL96	8801.656	8797.128
	A-815957	antis	F12	PSuCfacuUfuAfUfugagUfuUfcugugscsc	7579.797	7575.015

Upper case letter followed with f – 2'-F sugar modification; lower case letter – 2'-OMe sugar modification; s – phosphorothioate (PS) linkage; the structures for the prodrug Pmds and ligand L96 are the same as in Table 8 above.

Example 4. DTT reduction assay to check the cleavability of the cyclic phosphate prodrug analogues.

[0620] Reduction of the 5'-cyclic modified phosphate prodrugs by dithiothreitol (DTT) to 5' phosphate or 5' phosphorothioate was explored by incubating 100 μM modified oligonucleotide (23-nt length) with 100 mM DTT in 1X PBS. The amount of full-length (non-reduced) oligonucleotide was observed via LCMS analysis (either Agilent Single-Quad MS or Novatia HTSC) up to 72 hours post incubation.

Table 11. MS data from Novatia LCMS

Oligo ID	Sequence	% Full length at 0h	MW at 0h	% Full length at 0h	MW at 24h	% Full length at 0h	MW at 72h
A-515489	(Pmd)uCfacuUfuAfUfugag UfuUfcugugscsc	100	7709.1	100	7709.0	100	7709.2
A-515491	(Pmds)uCfacuUfuAfUfugag UfuUfcugugscsc	100	7725.4	100	7725.4	100	7725.7

Table 12. MS data from Agilent LCMS

Oligo ID	Sequence	% Full-length at 0h	MW at 0h	% Full length at 24h	MW at 24h	MW (5' PS) at 24h
A-1875173	(Cymd)usCfsacuUfuAfUfug agUfuUfcugugscsc	100	7721.32	100	7721.09	
A-1875172	(Cymds)usCfsacuUfuAfUfu gagUfuUfcugugscsc	100	7705.13	100	7705.11	
A-1875175	(Pd)usCfsacuUfuAfUfugag UfuUfcugugscsc	100	7744.6	<100	7744.4	7608.67 (~10-20%)
A-1875174	(Pds)usCfsacuUfuAfUfugag UfuUfcugugscsc	100	7728.37	100	7728.37	
A-1875179	(Pmmd)usCfsacuUfuAfUfug agUfuUfcugugscsc	100	7608.88	58	7755.03	7608.88 (42%)
A-1875178	(Pmmds)usCfsacuUfuAfUfu gagUfuUfcugugscsc	100	7739.01	14	7739.94	7592.83 (86%)
A-1875180	(Ptmd)usCfsacuUfuAfUfuga gUfuUfcugugscsc	100	7753.11	100	7753.16	
A-1875181	(Ptmds)usCfsacuUfuAfUfug agUfuUfcugugscsc	100	7769.1	100	7769.02	

Upper case letter followed with f – 2'-F sugar modification; lower case letter – 2'-OMe sugar modification; s – phosphorothioate (PS) linkage; the structures for the prodrugs are the same as in Table 8 above.

[0621] The representative LCMS spectra of the oligonucleotides tested in the DTT reduction assay are shown in **Figures 4A-J**.

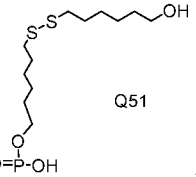
Example 5. Glutathione assay to check the cleavability of cyclic prodrug analogues.

[0622] Modified oligonucleotide (11-nt or 23-nt length) was added at 100 µM to a solution of 250 µg (6.25U/mL) glutathione-S-transferase from equine liver (GST) (Sigma Cat. No. G6511) and 0.1 mg/mL NADPH (Sigma Cat. No. 481973) in 0.1 M Tris pH 7.2. Glutathione (GSH) (MP Biomedicals, Inc. Cat. No. 101814#) was added to the mixture for a final concentration of 10 mM. Immediately after addition of GSH, sample was injected onto a Dionex DNAPac PA200 column (4x250mm) at 30 °C and run on an anion exchange gradient of 35-65% (20mM Sodium Phosphate, 10-15% CH_3CN, 1M Sodium Bromide pH11) at 1 mL/min for 6.5 minutes.

[0623] Glutathione-mediated cleavage kinetics were monitored every hour for 24 hours. The area under the main peak for each hour was normalized to the area from the 0 h time point (first injection). First-order decay kinetics were used to calculate half-lives. A control sequence containing modified oligonucleotide (23-nt length) with 5' Thiol modifier C6 (Glen Research Cat.No. 10-1936-02) between N6 and N7 was run each day of assay run. A second control sequence containing modified oligonucleotide (23-nt length) with the same 5' thiol modifier C6 at N1 was also run once per set of sequences. Half-lives were reported relative to half-life of control sequence. Glutathione and GST were prepared as stocks of 100 mM and 10 mg/mL in water, respectively, and aliquoted into 1 mL tubes and stored at -80 °C. A new aliquot was used for every day the assay was run.

Table 13. Half-life of oligonucleotides 5' modified phosphate prodrug after incubating with glutathione

Oligo ID	Sequence	Half-Life (h)
A-515432	**(Pmd)**dTdTdTdTdTdTdTdTdTdT	>24
A-515433	**(Pmds)**dTdTdTdTdTdTdTdTdTdT	>24
A-801703 (Control 1)	Q51uUfaUfaGfaGfcAfagaAfcAfcUfgUfuuu	<1
A-801704 (Control 2)	uUfaUfaGfQ51GfcAfagaAfcAfcUfgUfuuu	4.2
A-1875173	(Cymd)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24
A-1875172	(Cymds)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24
A-1875175	(Pd)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24
A-1875174	(Pds)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24
A-1875179	(Pmmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24
A-1875178	(Pmmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24
A-1875180	(Ptmd)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24
A-1875181	(Ptmds)usCfsacuUfuAfUfugagUfuUfcugugscsc	>24

Upper case letter followed with f – 2'-deoxy-2'-fluoro (2'-F) sugar modification; lower case letter – 2'-*O*-methyl (2'-OMe) sugar modification; s – phosphorothioate (PS) linkage; the structures for the prodrug are the same as in Table 9 above; Q51 – 

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Example 6. *In vivo* evaluation of siRNA duplexes containing modified phosphate prodrugs at the 5' end

[0624] *In vivo* study procedure: C57bl6 female mice (n=3/group) were dosed with 0.3 mg/mL of siRNA duplex containing the modified phosphate prodrugs at the 5' end in Table 14. Serum was collected at days 11, 22 and 35 days pos-dose and analyzed via ELISA to

determine relative F12 protein levels. The results are shown in **Figure 5**.

Table 14. siRNA duplexes used in the *in vivo* evaluation shown in Figure 5

Duplex ID	Oligo ID	Strand	Target	Oligo Seq	Molecular Weight	Molecular Weight found
AD-73603	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-147465	antis	F12	usCfsacuUfuAfUfugag UfuUfcugugscsc	7514.875	7511.025
AD-74843	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-150219	antis	F12	VPusCfsacuUfuAfUfug agUfuUfcugugscsc	7591.874	7587.005
AD-291896	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-447595	antis	F12	VPuCfacuUfuAfUfugag UfuUfcugugscsc	7558.743	7555.05
AD-326758	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-515487	antis	F12	uCfacuUfuAfUfugagUf uUfcugugscsc	7482.744	7479.071
AD-326756	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-515488	antis	F12	(Pmd)usCfsacuUfuAfUf ugagUfuUfcugugscsc	7744.095	7738.993
AD-326757	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-515489	antis	F12	(Pmd)uCfacuUfuAfUfu gagUfuUfcugugscsc	7711.964	7707.039
AD-326759	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-515490	antis	F12	(Pmds)usCfsacuUfuAfU fugagUfuUfcugugscsc	7760.161	7754.97
AD-326760	A-147464	sense	F12	csascagaAfaCfUfCfaaua aagugaL96	8801.656	8797.128
	A-515491	antis	F12	(Pmds)uCfacuUfuAfUfu gagUfuUfcugugscsc	7728.03	7723.016

Upper case letter followed with f – 2'-F sugar modification; lower case letter – 2'-OMe sugar modification; s – phosphorothioate (PS) linkage; the structures for the prodrug Pmds and ligand L96 are the same as in Table 8 above.

[0625] *In vivo* study procedure: C57bl6 female mice (n=3/group) were dosed with either 0.1 mg/mL or 0.3 mg/mL of siRNA duplex containing the modified phosphate prodrugs at the 5' end in Table 15. Serum was collected at days 11, 22 and 35 days pos-dose and analyzed via ELISA to determine relative F12 protein levels. The results are shown in

Figure 6.

Table 15. siRNA Duplexes used for *in vivo* evaluation in **Figure 6**

Duplex ID	Sense Strand Sequence (5'-3')	Antisense Strand Sequence (5'-3')
AD-73603	csascagaAfaCfUfCfaauaaagugaL96	usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-291896	csascagaAfaCfUfCfaauaaagugaL96	VPuCfacuUfuAfUfugagUfuUfcugugscsc
AD-1023152	csascagaAfaCfUfCfaauaaagugaL96	(Pmmds)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1144832	csascagaAfaCfUfCfaauaaagugaL96	(Pmmds)uCfacuUfuAfUfugagUfuUfcugugscsc

Upper case letter followed with f – 2'-F sugar modification; lower case letter – 2'-OMe sugar modification; s – phosphorothioate (PS) linkage; the structures for the prodrug Pmds and ligand L96 are the same as in Table 8 above.

Example 7. *In vivo* metabolic stability and determination of 5'-phosphate

[0626] Metabolic stability study procedure: C57bl6 female mice (n=2/group) Mice were dosed with 10 mg/kg siRNA duplex containing the modified phosphate prodrugs at the 5' end in Table 16. Livers were collected 5 days post-dose and analyzed via LC-MS.

Table 16. siRNA Duplexes used for metabolic evaluation

Duplex ID	Sense Strand Sequence (5'-3')	Antisense Strand Sequence (5'-3')
AD-326760	csascagaAfaCfUfCfaauaaagugaL96	(Pmds)uCfacuUfuAfUfugagUfuUfcugugscsc
AD-1023152	csascagaAfaCfUfCfaauaaagugaL96	(Pmmds)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023155	csascagaAfaCfUfCfaauaaagugaL96	(Cymds)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023156	csascagaAfaCfUfCfaauaaagugaL96	(Pds)usCfsacuUfuAfUfugagUfuUfcugugscsc
AD-1023157	csascagaAfaCfUfCfaauaaagugaL96	(Ptmds)usCfsacuUfuAfUfugagUfuUfcugugscsc

Upper case letter followed with f – 2'-F sugar modification; lower case letter – 2'-OMe sugar modification; s – phosphorothioate (PS) linkage; the structures for the prodrugs are the same as in Table 8 above.

[0627] The results of the metabolic stability study are shown in Tables 17 and 18.

Table 17. siRNA metabolites found after *in vivo* study

Duplex ID	Strand (5'→3')	Metabolites (%)									
		FL	(pd)N1	N3N4	N5N6	N13N14	N18N19	N20N21	N21L96	N22N23	(pd)N1+ N22N23
AD-326760	Sense	98					1	1			
	Antisense	72				1				28	
AD-1023155	Sense	97						1	2		
	Antisense	70	6							21	2
AD-1023156	Sense	100									
	Antisense	64	12							18	6
AD-1023152	Sense	96					1	1	2		
	Antisense	72			2					25	1
AD-1023157	Sense	90		2			2	2	4		
	Antisense	71			2					27	

Sense (5'→3') = N1N2N3N4N5N6N7N8N9N10N11N12N13N14N15N16N17N18N19N20N21L96
Antisense (5'→3') = (pd)N1N2N3N4N5N6N7N8N9N10N11N12N13N14N15N16N17N18N19N20N21N22N23

FL = (Full Length Strand)
(pd) = prodrug

Table 18. % of 5'-phosphate, thiophosphate, or hydroxy group found after 5 days in mice on antisense strand

Duplex ID	5'PO (%)	5'PS (%)	5'OH (%)
AD-326760	0	0	5
AD-1023155	0	0	8
AD-1023156	0	0	18
AD-1023152	1.6	1.3	1
AD-1023157	0	0	0

[0628] The possible *in vivo* cytosolic unmasking mechanism of the 5' cyclic disulfide modified phosphate prodrugs to reveal 5'-phosphate is shown in Figure 7.

Example 8: *In vivo* evaluation of siRNA duplexes containing modified phosphate prodrugs at the 5' end in CNS

[0629] *In vivo* study procedure for CNS (IT- Intrathecal administration): Female rat (n=3/group) were dosed with 0.1 mg/rat, 0.3 mg/rat, or 0.9 mg/rat of SOD1 siRNA duplex containing the modified phosphate prodrugs at the 5' end in Table 19. Brain was collected after 14 or 84 days of IT administration and dissected into different regions for qPCR analysis to determine the relative SOD1 mRNA levels. The results are shown in **Figures 8-13**.

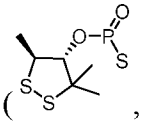
[0630] *In vivo* study procedure for CNS (ICV- intracranial ventricular administration): C57bl6 female mice (n=4/group) were dosed with 100 µg of SOD1 siRNA duplex containing the modified phosphate prodrugs at the 5' end in Table 19. Brain was collected after 7 days of ICV administration and the right hemisphere was used for qPCR analysis to determine the relative SOD1 mRNA levels. The results are shown in **Figure 14.**

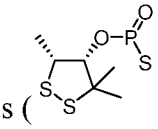
Table 19. siRNAs for CNS studies

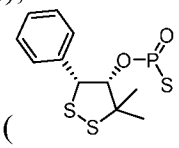
Duplex Id	Oligo Id	Strand	Target	Oligo Sequence	Molecular Weight	Exact Mass
401824	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	444402	antis		VPusUfsuagAfgUfGfaggaUfuAfaaaugsasg	7851.16	7847.15
401825	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	268862	antis		usUfsuagAfgUfGfaggaUfuAfaaaugsasg	7775.16	7771.18
1548732	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	2717577	antis		(Pmmds)usUfsuagAfgUfGfaggaUfuAfaaaugsasg	8017.47	8013.14
1548737	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	2717578	antis		(cPmmds)usUfsuagAfgUfGfaggaUfuAfaaaugsasg	8017.47	8013.14
1548733	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	2717579	antis		(Pds)usUfsuagAfgUfGfaggaUfuAfaaaugsasg	8005.41	8001.11
1548734	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	2717580	antis		(Pmmds)UsUfsuagAfgUfGfaggaUfuAfaaaugsasg	8003.44	7999.13
1548735	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	2717581	antis		(Pmmds)Us(Ufms)uagAfgUfGfaggaUfuAfaaaugsasg	8017.47	8013.14
1548736	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	2717582	antis		(Pmmds)(URs)(Ufms)uagAfgUfGfaggaUfuAfaaaugsasg	8013.14	8013.14
1700788	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	3019034	antis		(PdAr1s)usUfsuagAfgUfGfaggaUfuAfaaaugsasg	8079.53	8075.16
1700790	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25

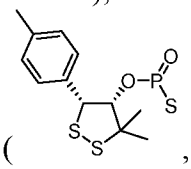
	3019035	antis		(PdAr3s)usUfsuagAfgUfGfaggaUfuAfa aaugsasg	8093.56	8089.17
1700789	637448	sense	SOD1	csasuuu(Uhd)AfaUfCfCfucacucuasasa	7043.98	7040.25
	3019036	antis		(PdAr5s)usUfsuagAfgUfGfaggaUfuAfa aaugsasg	8109.56	8105.17

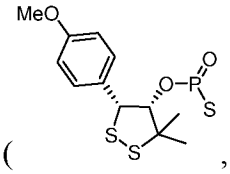
Upper case letter followed with f – 2'-deoxy-2'-fluoro (2'-F) sugar modification; lower case letter – 2'-*O*-methyl (2'-OMe) sugar modification; s – phosphorothioate (PS) linkage; Uhd: 2'-*O*-hexadecyl uridine (2'-C16); VP – vinyl phosphonate; the prodrugs:

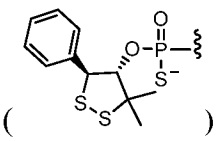
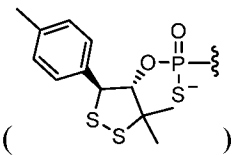
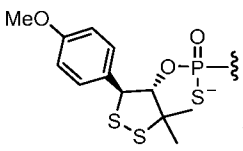
Pmmds ( , ((4SR,5SR)-3,3,5-trimethyl-1,2-dithiolan-4-ol) phosphodiester);

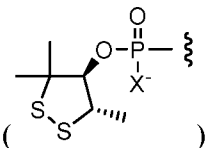
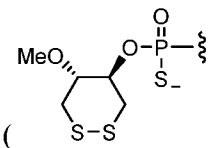
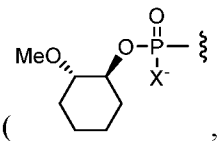
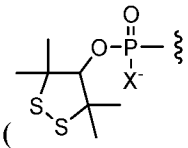
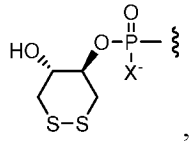
cPmmds ( , ((4SR,5RS)-3,3,5-trimethyl-1,2-dithiolan-4-ol) phosphodiester (Cis Pmmds));

PdAr1s ( , ((4SR,5RS)-5-phenyl-3,3-dimethyl-1,2-dithiolan-4-ol) phosphodiester);

PdAr3s ( , ((4SR,5RS)-5-(4-methylphenyl)-3,3-dimethyl-1,2-dithiolan-4-ol) phosphodiester);

PdAr5s ( , ((4SR,5RS)-5-(4-methoxyphenyl)-3,3-dimethyl-1,2-dithiolan-4-ol) phosphodiester);

PdAr2s (); PdAr4s (); PdAr6s ();

Pmmd/Pmmds (); Pmds (); Cymd/Cymds ( , X is O/S); Ptmd/Ptmds ( , X is O/S), Pd/Pds ( , X is O/S),.

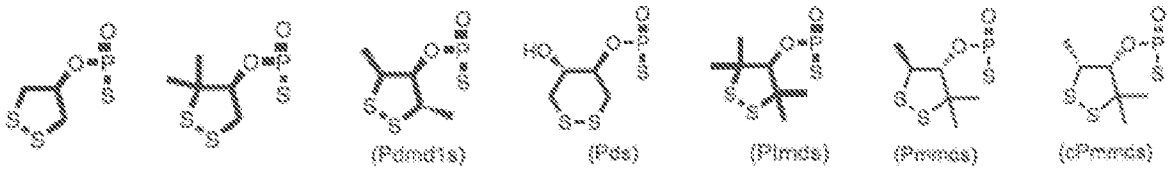
[0631] As shown in Figures 8-11, the siRNA duplex containing Pmmds and cPmmds prodrugs at the 5' end displayed similar activity and duration as the siRNA duplex containing

5'-VP control in CNS tissues.

[0632] As shown in Figure 12-13, the siRNA duplex containing PdAr1s, PdAr3s, and PdAr5s prodrugs at the 5' end displayed better or at least comparable activity as compared to the siRNA duplex containing 5'-VP control in CNS tissues.

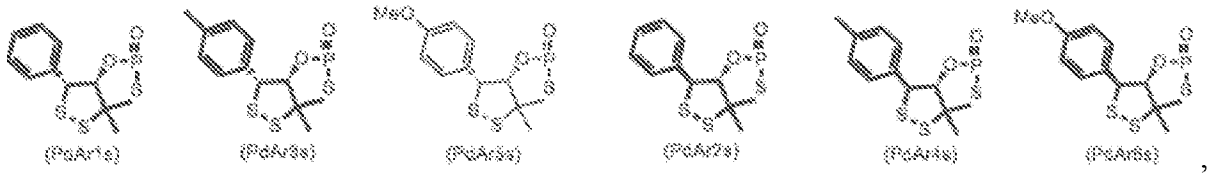
[0633] Overall, metabolically stable 5'-phosphate mimic such as 5'-VP could improve siRNA activity in extrahepatic tissues with less efficient endogenous 5'-phosphorylation of modified siRNA. The novel 5' modified phosphate prodrug described herein showed stability in plasma and endosomal environment. These 5' modified phosphate prodrugs unmasked in cytosol to reveal f natural 5'-phosphate (or phosphorothioate) required for efficient RISC loading. The siRNAs containing novel 5' modified phosphate prodrugs at the 5' end displayed activity comparable to or even better than that of siRNAs containing a stable 5'-phosphate mimic design, such as 5'-VP.

[0634] For instance, the activity of the siRNAs containing the following list of 5' modified phosphate prodrugs,



,

were generally comparable to the activity of siRNAs containing 5'-VP. The siRNAs containing the following list of 5' modified phosphate prodrugs,



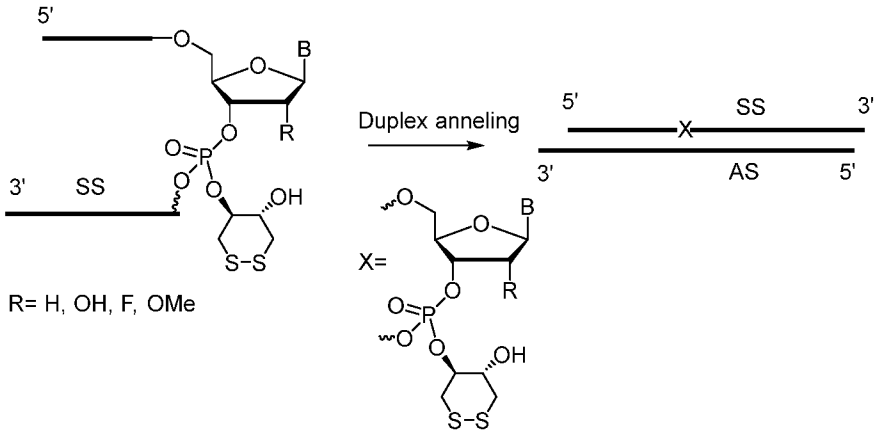
,

generally have an improved stability than that of siRNAs containing 5'-VP and have a better or comparable activity than that of siRNAs containing 5'-VP.

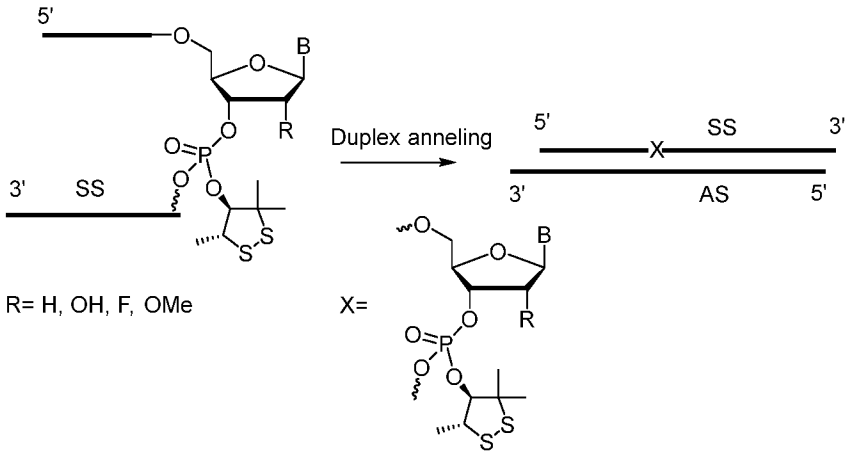
Example 9. Introduction of the modified phosphate prodrugs for masking internucleotide phosphate linkages to mask the charge

[0635] Different cyclic phosphate prodrug derivatives can be introduced to the phosphate group as a temporary protecting group to any internucleotide phosphate group on either the sense or antisense strand or both the sense and antisense strands, as shown in the Schemes 14-19.

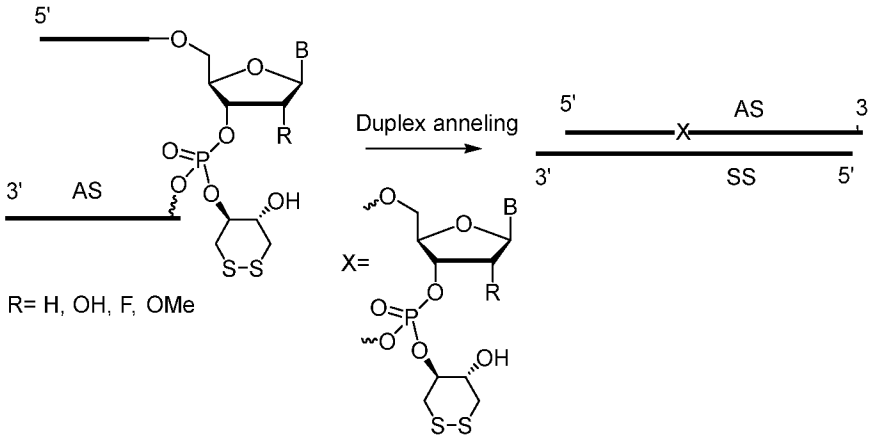
Scheme 14



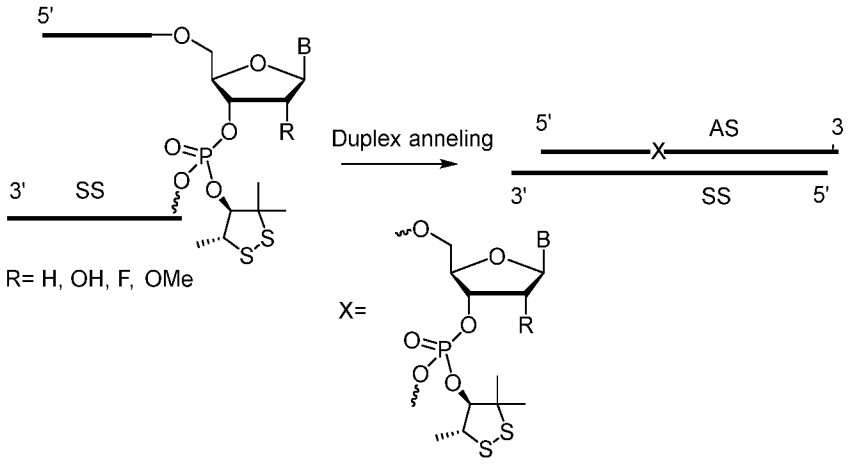
Scheme 15



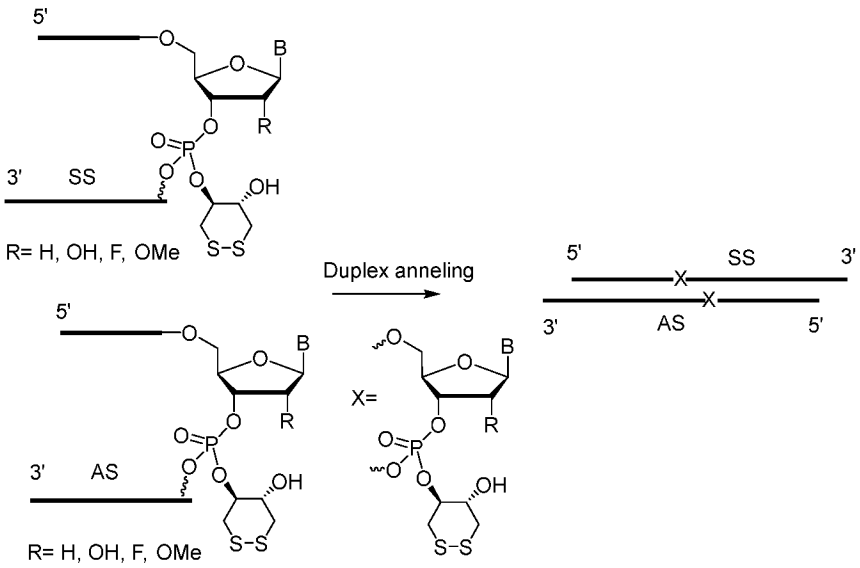
Scheme 16



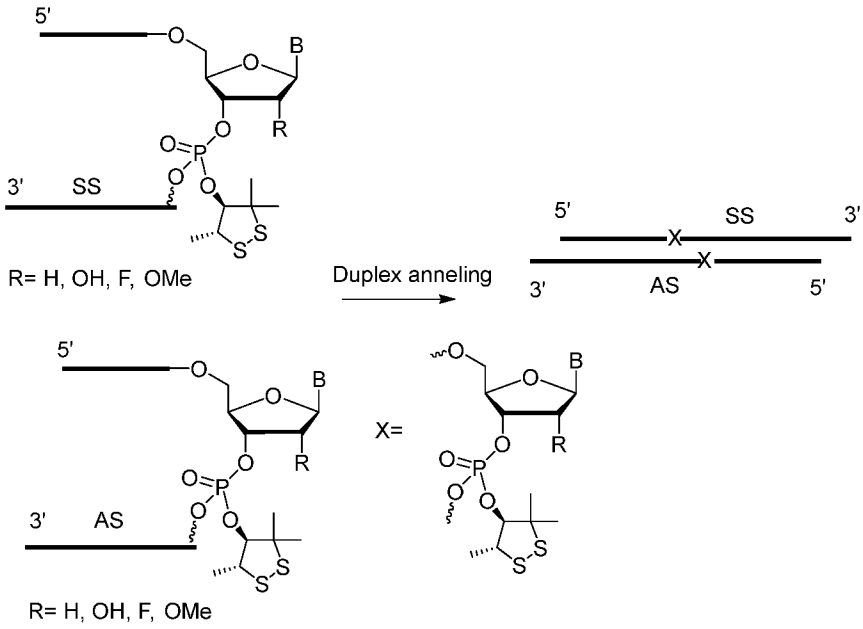
Scheme 17



Scheme 18



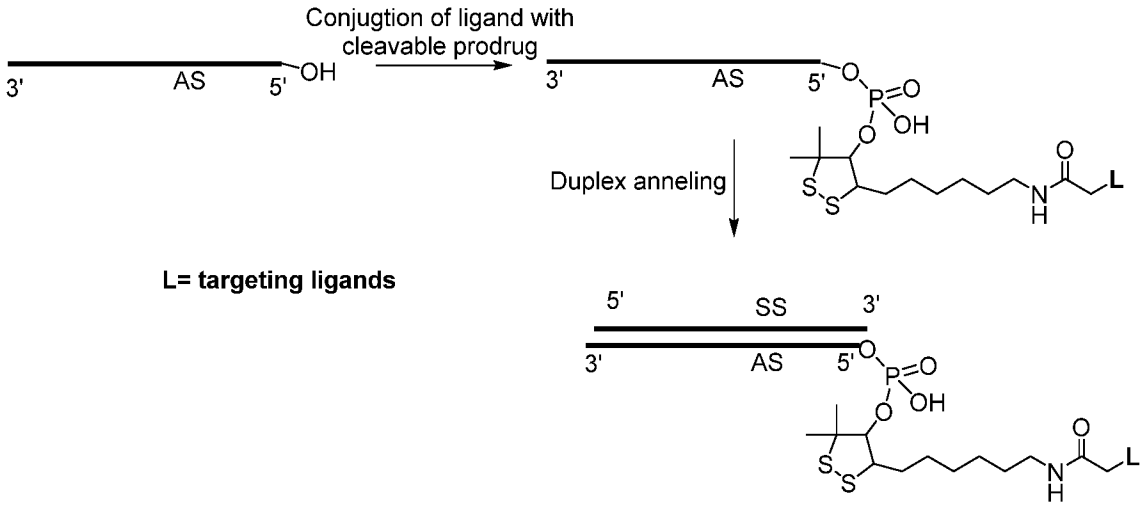
Scheme 19



Example 10. Using the modified phosphate prodrugs to generate cleavable siRNA conjugates having different targeting ligands

[0636] Different targeting ligands can be introduced into a siRNA duplex through the cyclic phosphate derivatives as shown in Scheme 20. These derivatives will be cleaved off after the siRNA enters into cytosol.

Scheme 20

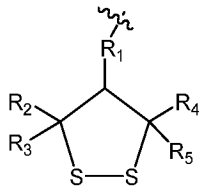


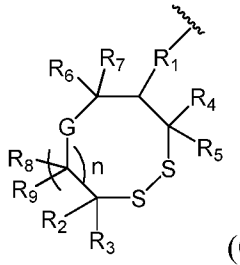
We claim:

1. A compound comprising a structure of formula (I):

cyclic disulfide moiety — phosphorus coupling group (I)

wherein the cyclic disulfide moiety has the structure of:



(C-I) or


(C-II),

wherein:

R_1 is O or S, and is bonded to the P atom of the phosphorus coupling group;

R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

R_3 and R_5 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring;

G is O, N(R'), S, or $C(R^{14})(R^{15})$;

n is an integer of 0-6;

R^{13} is independently for each occurrence H, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, alkylcarbonyl, or arylcarbonyl, each of which can be optionally substituted with one or more R^{sub} groups;

R^{14}, R^{15}, and R^{16} are each independently H, halo, haloalkyl, alkyl, alkaryl, aryl, heteroaryl, aralkyl, hydroxy, alkyloxy, aryloxy, N(R')(R");

R' and R" are each independently H, alkyl, alkenyl, alkynyl, aryl, hydroxy, alkyloxy, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, or ω-hydroxy alkynyl, each of which can be optionally substituted with one or more R^{sub} groups; and

R^{sub} is independently for each occurrence halo, haloalkyl, alkyl, alkaryl, aryl, aralkyl, hydroxy, alkyloxy, aryloxy, oxo, nitro, amino, acylamino, alkylcarbamoyl, arylcarbamoyl,

alkylamino, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, arylcarbonyl, acyloxy, cyano, or ureido.

2. The compound of claim 1, wherein in the cyclic disulfide moiety:

R_1 is O;

G is CH_2;

n is 0 or 1;

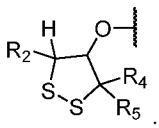
R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or C_1-C_6 alkylene-OR^{13}, N(R')(R") or C_1-C_6 alkylene-N(R')(R"), C_1-C_6 alkyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

R_3 and R_5 are each independently H, halo, OR^{13} or C_1-C_6 alkylene-OR^{13}, N(R')(R") or C_1-C_6 alkylene-N(R')(R"), C_1-C_6 alkyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring of 6-8 atoms;

R^{13} is independently for each occurrence H, C_1-C_6 alkyl, aryl, alkylcarbonyl, or arylcarbonyl; and

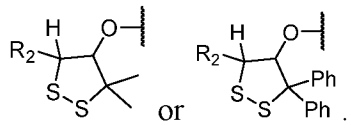
R' and R" are each independently H or C_1-C_6 alkyl.

3. The compound of claim 1, wherein the cyclic disulfide moiety has the structure of:



.

4. The compound of claim 1, wherein the cyclic disulfide moiety has the structure of:



.

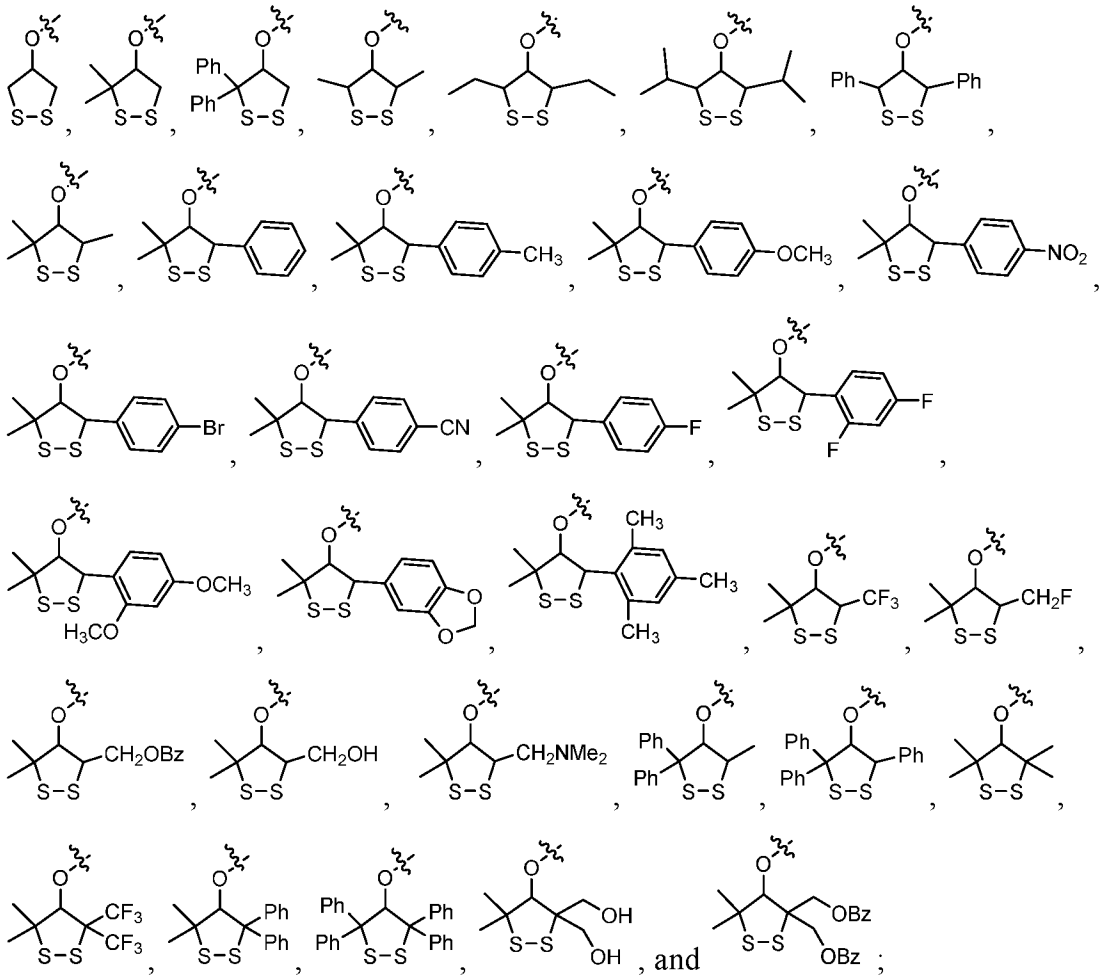
5. The compound of claim 3 or 4, wherein R_2 is optionally substituted aryl.

6. The compound of claim 3 or 4, wherein R_2 is optionally substituted C_1-$_6$ alkyl.

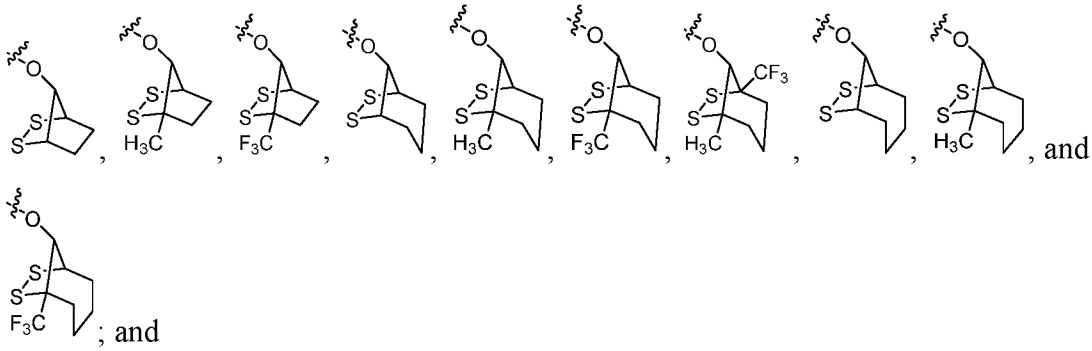
7. The compound of claim 1, wherein the cyclic disulfide moiety has the structure selected

from one of the following formula Ia), Ib), and II) groups:

Ia):

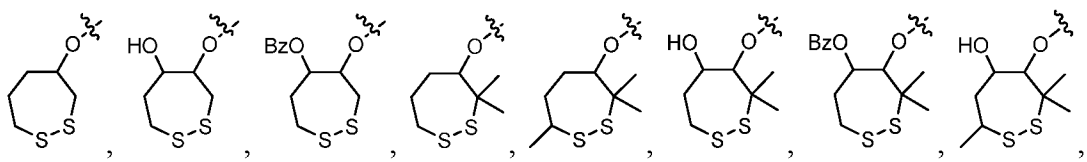


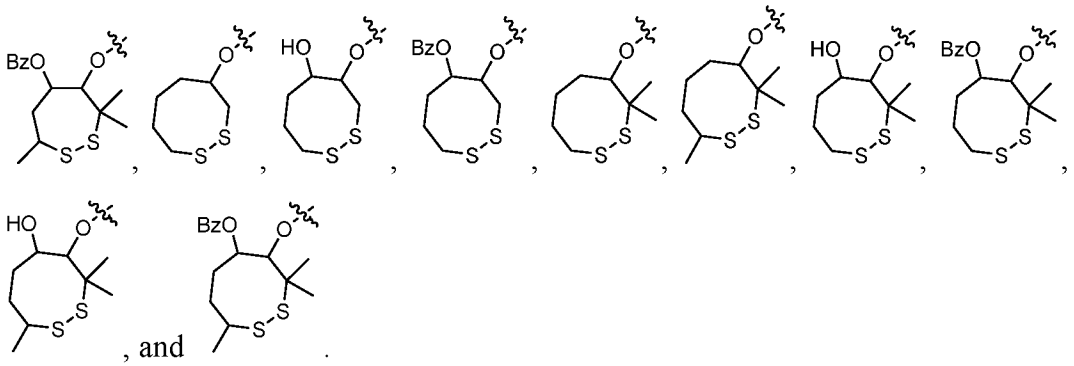
Ib):



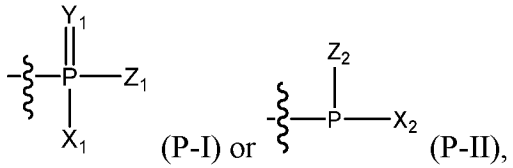
; and

II):





8. The compound of any one of claims 1-3, wherein the phosphorus coupling group has the structure of:



(P-I) or (P-II),

wherein:

X_1 and Z_1 are each independently H, OH, OM, OR^{13}, SH, SM, SR^{13}, C(O)H, S(O)H, or alkyl, each of which can be optionally substituted with one or more R^{sub} groups, N(R')(R"), $B(R^{13})_3$, BH_3^-, Se; or D-Q, wherein D is independently for each occurrence absent, O, S, N(R'), alkylene, each of which can be optionally substituted with one or more R^{sub} groups, and Q is independently for each occurrence a nucleoside or oligonucleotide;

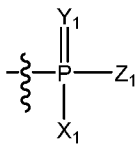
X_2 and Z_2 are each independently N(R')(R"), OR^{18}, or D-Q, wherein D is independently for each occurrence absent, O, S, N, N(R'), alkylene, each of which can be optionally substituted with one or more R^{sub} groups, and Q is independently for each occurrence a nucleoside or oligonucleotide,

Y_1 is S, O, or N(R');

M is an organic or inorganic cation; and

R^{18} is H or alkyl, optionally substituted with one or more R^{sub} groups.

9. The compound of any one of claims 1-3, wherein the phosphorus coupling group has the structure of



(P-I),

wherein:

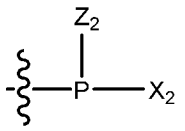
X_1 and Z_1 are each independently OH, OM, SH, SM, C(O)H, S(O)H, C_1-C_6 alkyl optionally substituted with one or more hydroxy or halo groups, or D-Q;

D is independently for each occurrence absent, O, S, NH, C_1-C_6 alkylene optionally substituted with one or more halo groups; and

Y_1 is S or O.

10. The compound of claim 9, wherein X_1 is OH or SH; and Z_1 is D-Q.

11. The compound of any one of claims 1-3, wherein the phosphorus coupling group has the structure of


(P-II),

wherein:

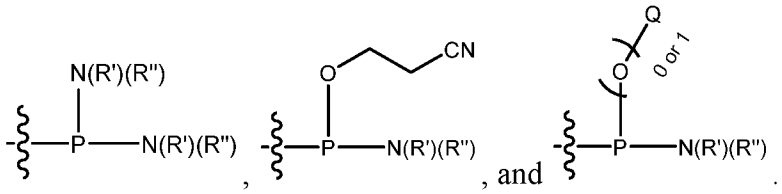
X_2 is N(R')(R");

Z_2 is X_2, OR^{18}, or D-Q;

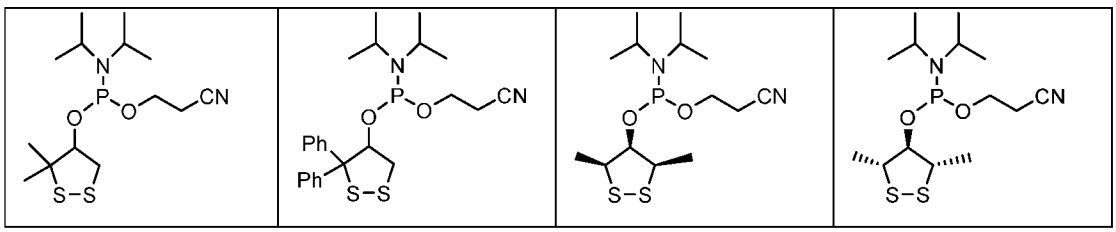
R^{18} is H or C_1-C_6 alkyl substituted with cyano; and

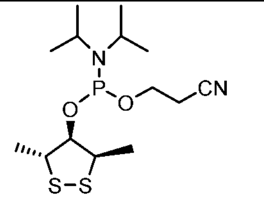
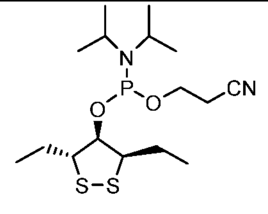
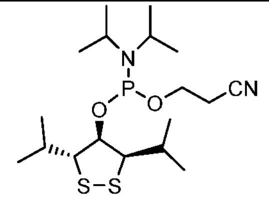
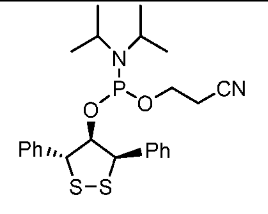
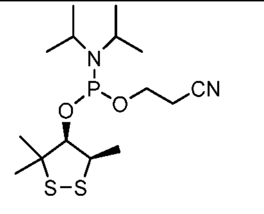
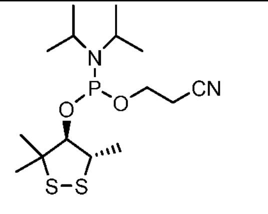
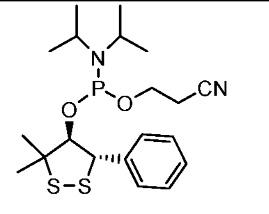
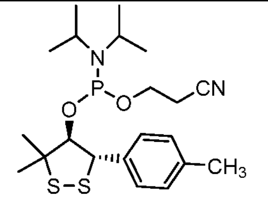
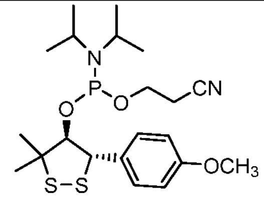
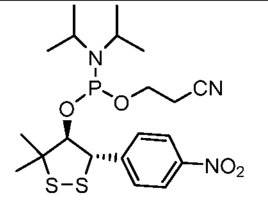
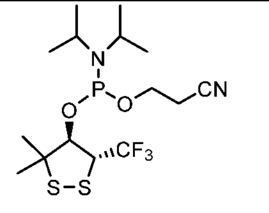
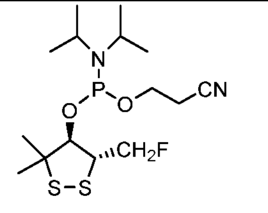
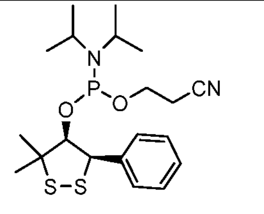
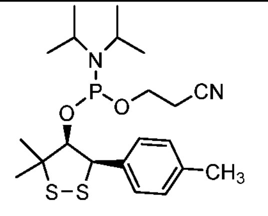
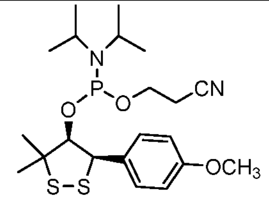
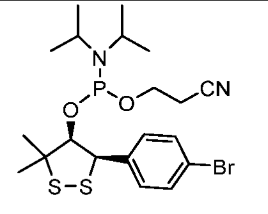
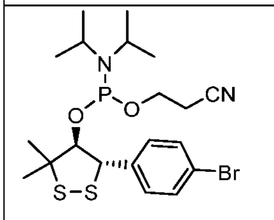
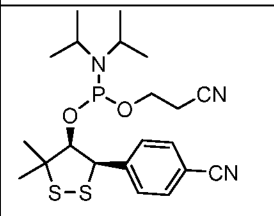
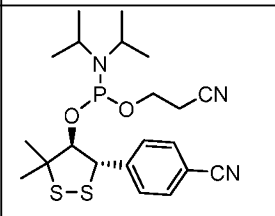
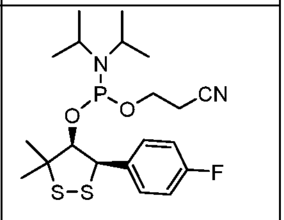
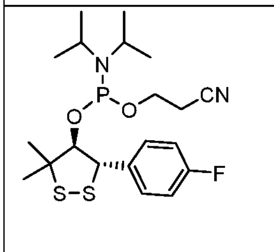
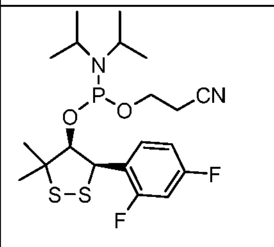
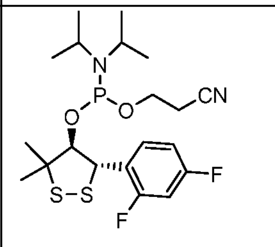
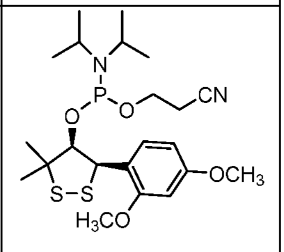
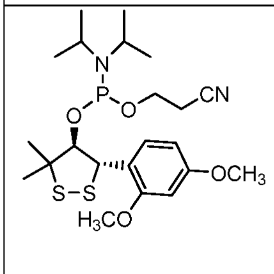
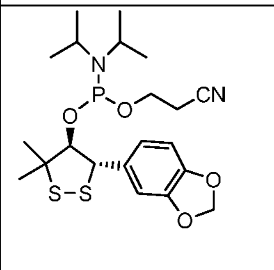
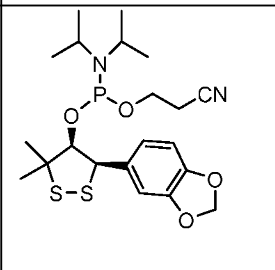
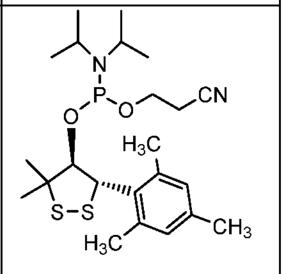
R' and R'' are each independent C_1-C_6 alkyl.

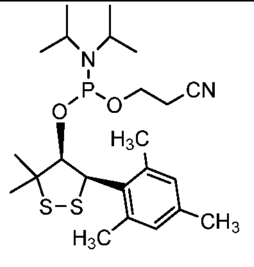
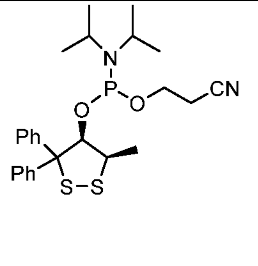
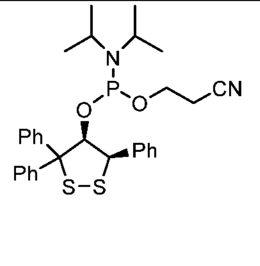
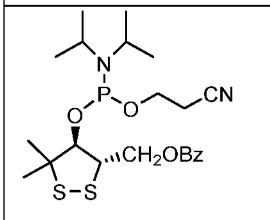
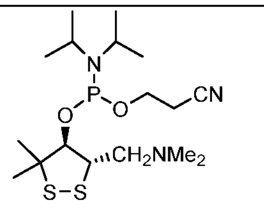
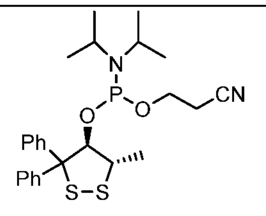
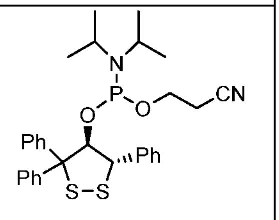
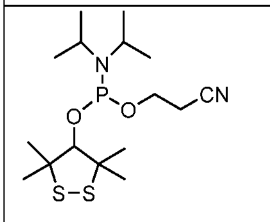
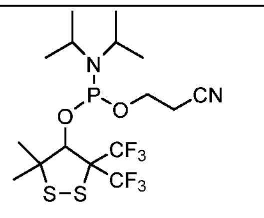
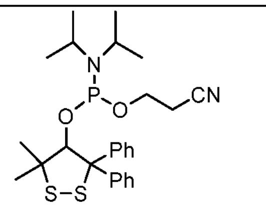
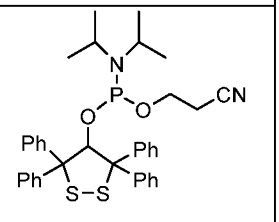
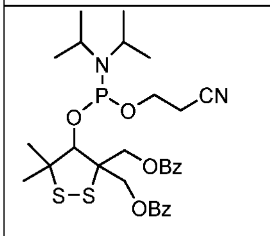
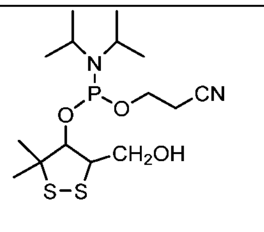
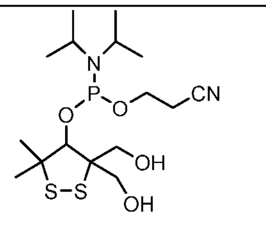
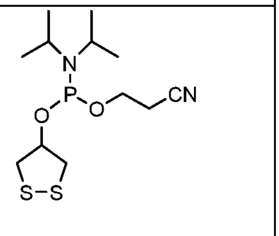
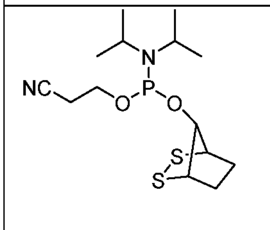
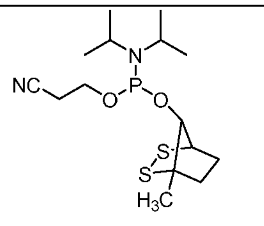
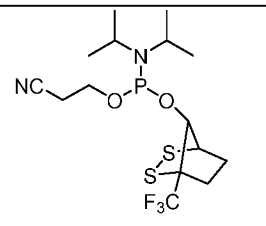
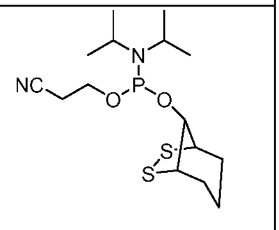
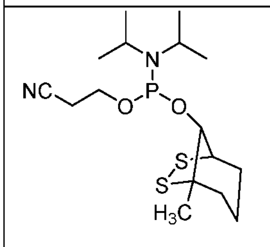
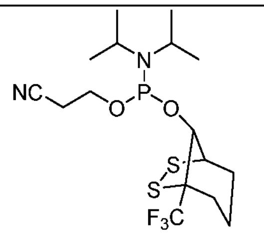
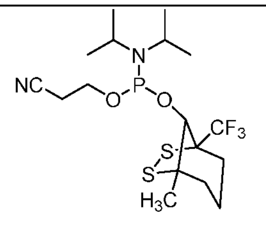
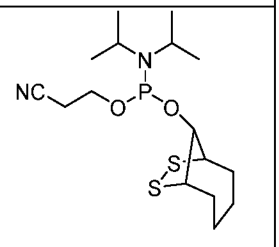
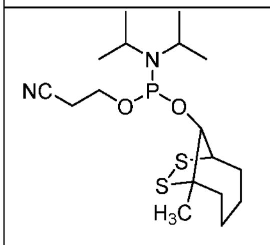
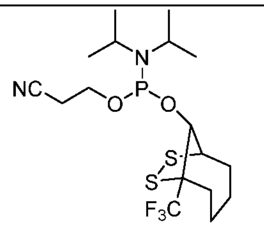
12. The compound of claim 11, wherein the phosphorus coupling group has a structure selected from the group consisting of

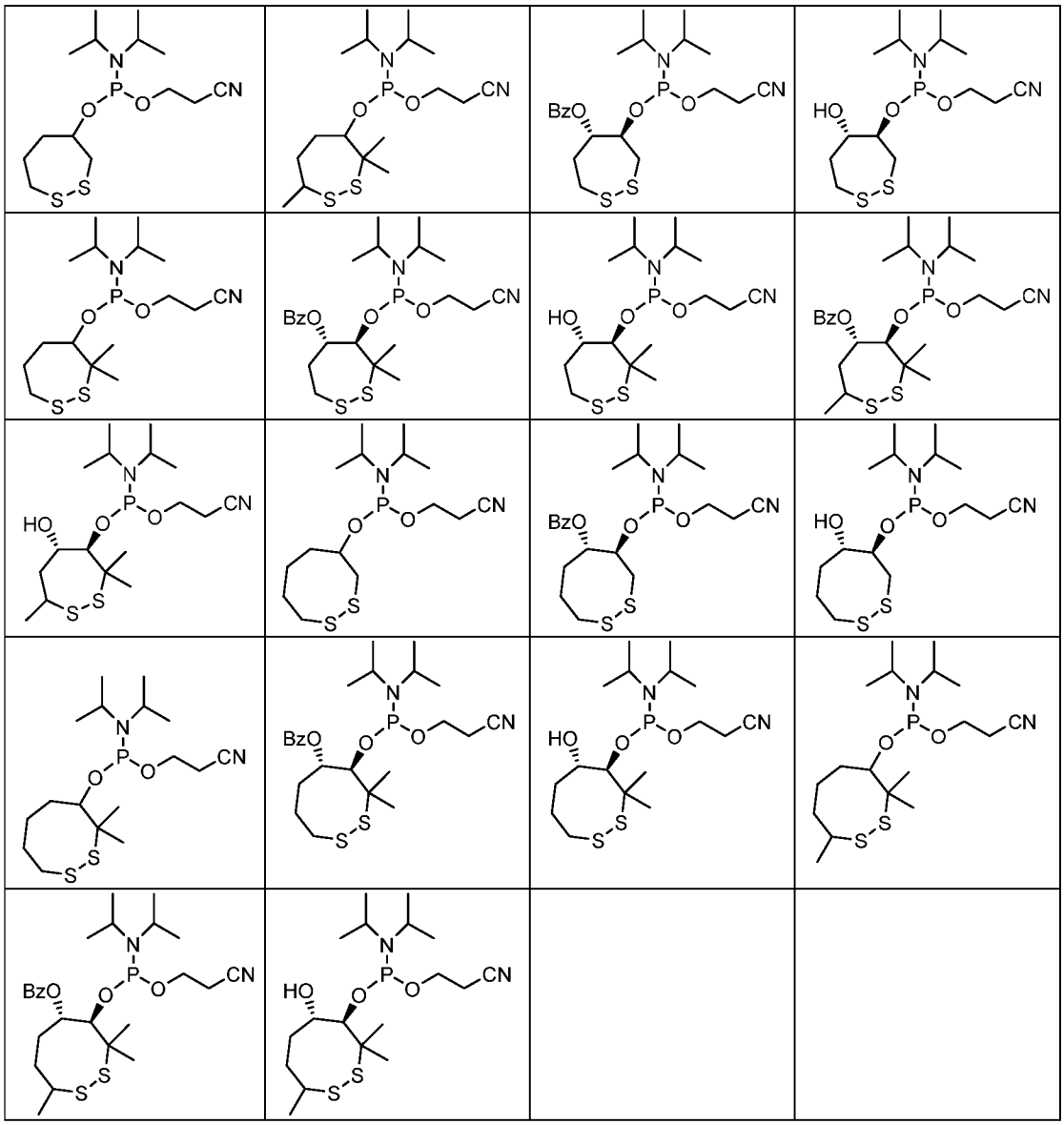

, and .

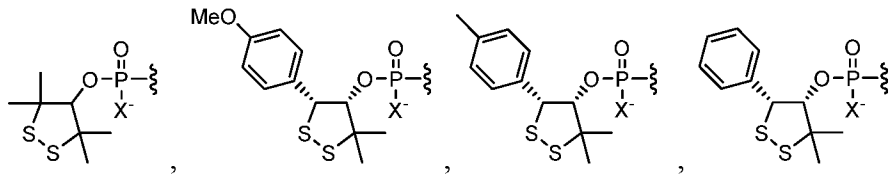
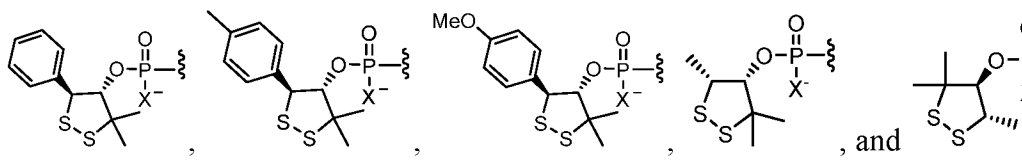
13. The compound of claim 1, wherein the compound has a structure selected from the group consisting of:





14. The compound of claim 8, wherein the phosphorus coupling group has the structure of (P-I), and the cyclic disulfide moiety—$P(Y_1)(X_1)$- has a structure selected from the group consisting of:  ,  , and , wherein X is O or S.

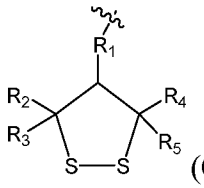
15. The compound of claim 1, wherein one or more ligands are connected to any one of R_2, R_3, R_4, R_5, R_6, R_7, R_8, and R_9 of the cyclic disulfide moiety, optionally via one or more linkers.

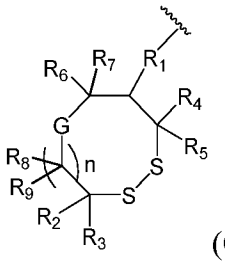
16. The compound of claim 15, wherein the ligand is selected from the group consisting of an antibody, a ligand-binding portion of a receptor, a ligand for a receptor, an aptamer, a carbohydrate-based ligand, a fatty acid, a lipoprotein, folate, thyrotropin, melanotropin, surfactant protein A, mucin, glycosylated polyaminoacids, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipophilic moiety, a cholesterol, a steroid, bile acid, vitamin B12, biotin, a fluorophore, and a peptide.

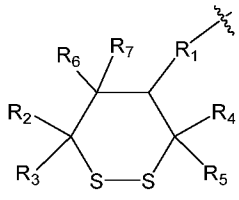
17. An oligonucleotide comprising one or more structures of formula (II):

cyclic disulfide moiety — P(Y)(X)-* (II)

wherein the cyclic disulfide moiety has the structure of:



(C-I),


(C-II), or


(C-III),

or a salt thereof,

wherein:

* represents the bond to the oligonucleotide,

Y is absent, =O, or =S, X is -OH, -SH or X', wherein X' is $-OR^{13}$ or $-SR^{13}$;

R_1 is O or S, and is bonded to the P atom of the -P(Y)(X)-* group;

R_2, R_4, R_6, R_7, R_8, and R_9 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups;

R_3 and R_5 are each independently H, halo, OR^{13} or alkylene-OR^{13}, N(R')(R") or alkylene-N(R')(R"), alkyl, $C(R^{14})(R^{15})(R^{16})$ or alkylene-$C(R^{14})(R^{15})(R^{16})$, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, each of which can be optionally substituted by one or more R^{sub} groups; or R_3 and R_5, together with the adjacent carbon atoms and the two sulfur atoms, form a second ring;

G is O, N(R'), S, or C(R^{14})(R^{15});

n is an integer of 0-6;

R^{13} is independently for each occurrence H, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, alkylcarbonyl, or arylcarbonyl, each of which can be optionally substituted with one or more R^{sub} groups;

R^{14}, R^{15}, and R^{16} are each independently H, halo, haloalkyl, alkyl, alkaryl, aryl, heteroaryl, aralkyl, hydroxy, alkyloxy, aryloxy, N(R')(R");

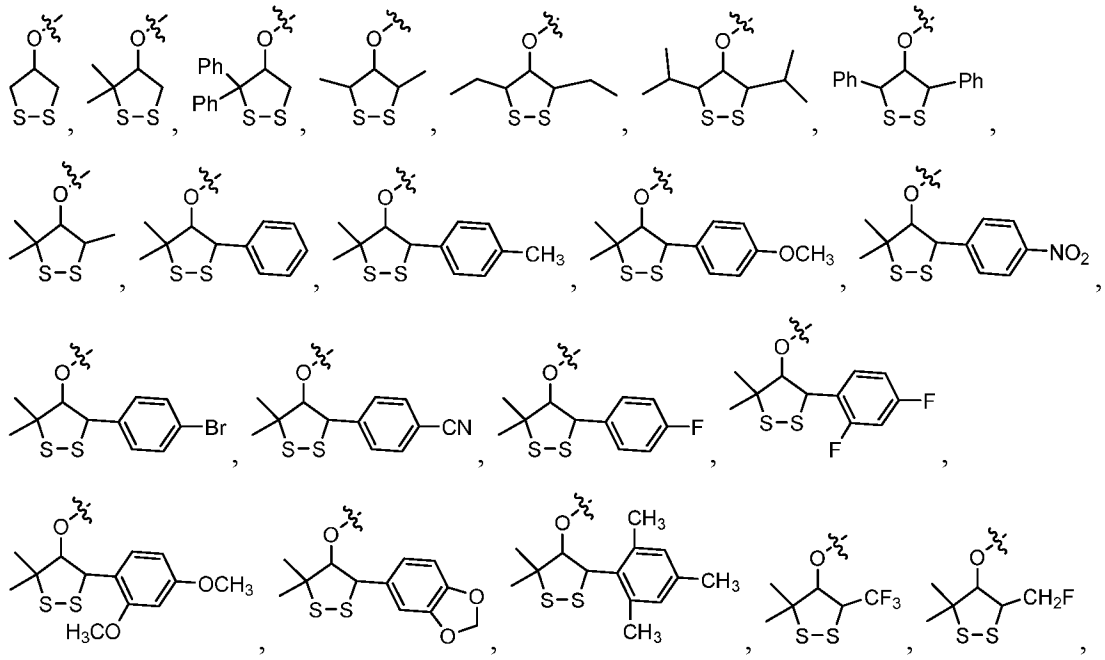
R' and R" are each independently H, alkyl, alkenyl, alkynyl, aryl, hydroxy, alkyloxy, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, or ω-hydroxy alkynyl, each of which can be optionally substituted with one or more R^{sub} groups; and

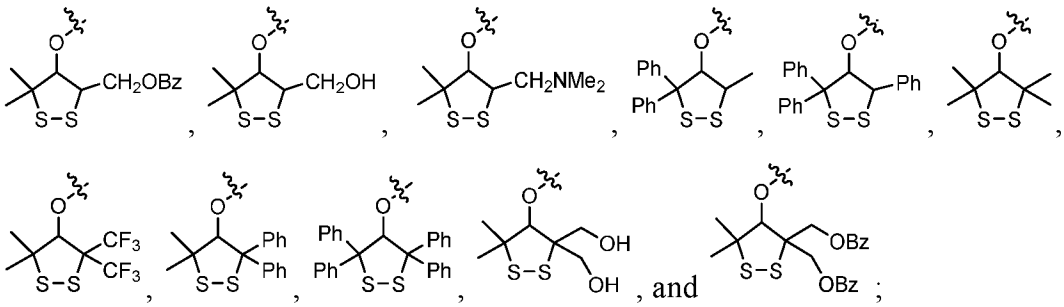
R^{sub} is independently for each occurrence halo, haloalkyl, alkyl, alkaryl, aryl, aralkyl, hydroxy, alkyloxy, aryloxy, oxo, nitro, amino, acylamino, alkylcarbamoyl, arylcarbamoyl, alkylamino, aminoalkyl, alkoxycarbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, arenesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, arylcarbonyl, acyloxy, cyano, or ureido;

wherein, when the cyclic disulfide moiety has the structure of formula (C-III), at least one cyclic disulfide moiety is connected at the 5' end of the nucleoside or oligonucleotide.

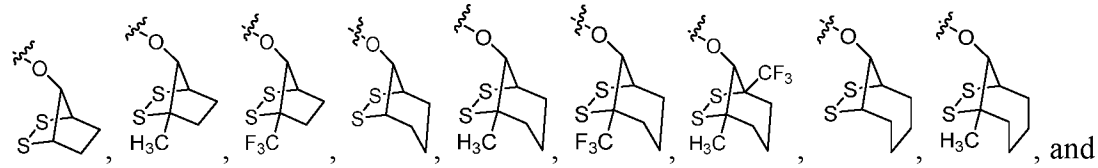
18. The oligonucleotide of claim 17, wherein the cyclic disulfide moiety has the structure selected from one of the following formula Ia), Ib), and II) groups:

Ia):



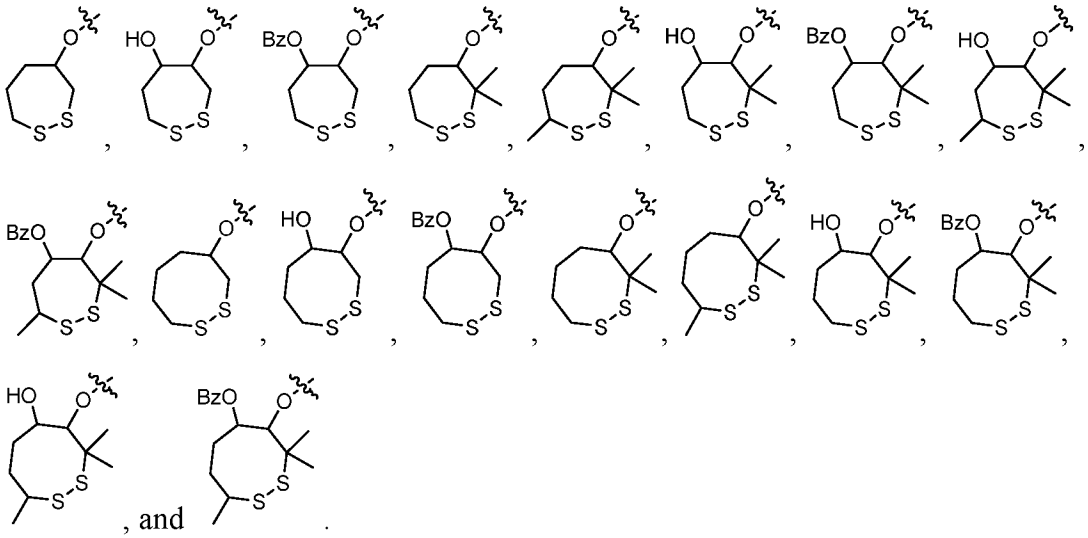


Ib):



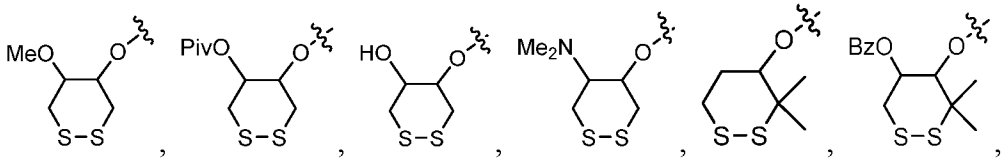
; and

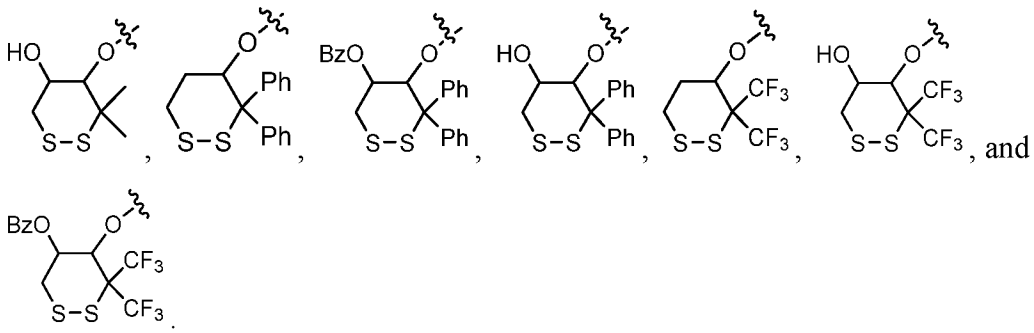
II):



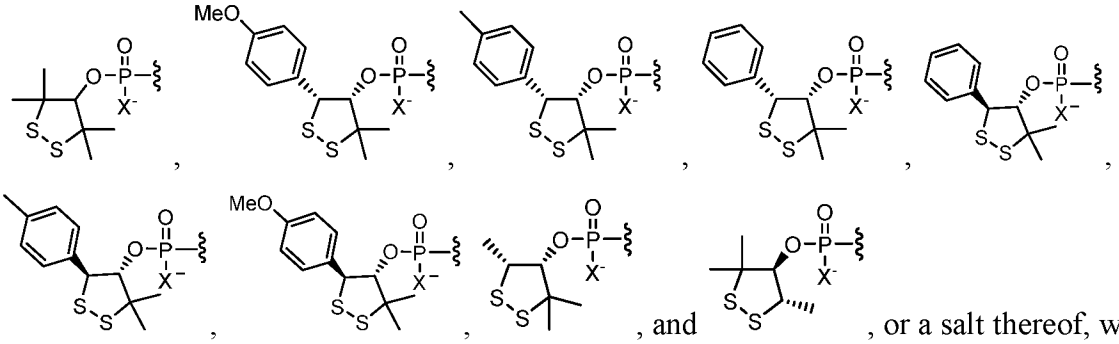
, and .

19. The oligonucleotide of claim 17, wherein the cyclic disulfide moiety has the structure selected from one of the following formula (III) groups:





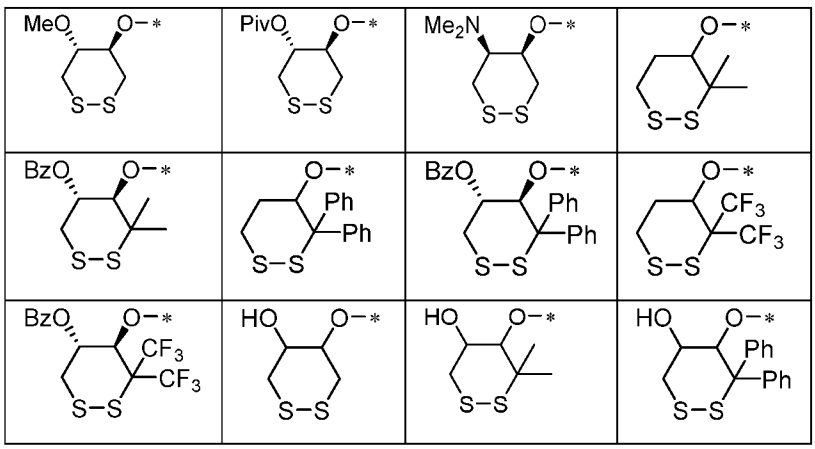
20. The oligonucleotide of claim 17, wherein the cyclic disulfide moiety-P(Y)(X)-* group has the structure selected from the group consisting of:

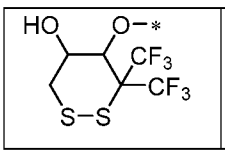


X is O or S.

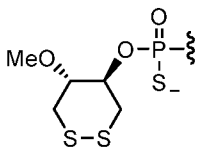
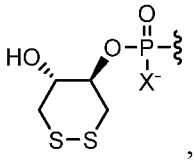
21. The oligonucleotide of any one of claims 17-20, comprising a structure having the formula: cyclic disulfide moiety —P(O)(SH)-*, cyclic disulfide moiety —P(O)(OH)-*, cyclic disulfide moiety —P(O)(OR^{13})-*, cyclic disulfide moiety —P(S)(OR^{13})-*, cyclic disulfide moiety —P(OR^{13})-*, or a salt thereof.

22. The oligonucleotide of claim 17, wherein the cyclic disulfide moiety has a structure selected from the group consisting of:



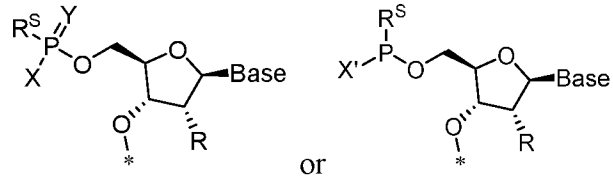
			

wherein * indicates the bond to the phosphorus atom of the -P(X)(Y)-* group.

23. The oligonucleotide of claim 22, wherein the cyclic disulfide moiety-P(Y)(X)-* has a structure selected from the group consisting of  and  , wherein X is O or S.

24. The oligonucleotide of claim 17, wherein the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end of the oligonucleotide.

25. The oligonucleotide of claim 24, wherein the first nucleotide at the 5'-end of the oligonucleotide has the structure:



or a salt thereof, wherein

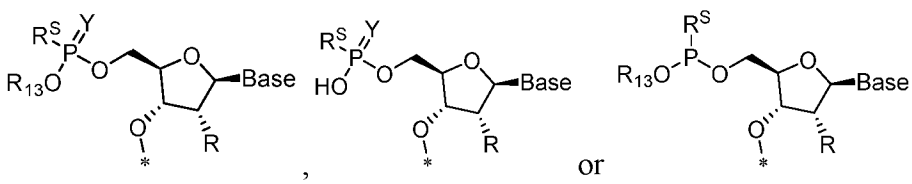
* represents a bond to the subsequent optionally modified internucleotide linkage;

Base is an optionally modified nucleobase;

R^S is the cyclic disulfide moiety; and

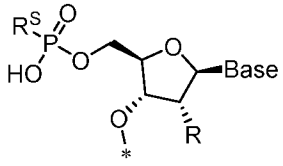
R is H, OH, O-methoxyalkyl, O-methyl, O-allyl, CH_2-allyl, fluoro, O-N-methylacetamido (O-NMA), O-dimethylaminoethoxyethyl (O-DMAEOE), O-aminopropyl (O-AP), or ara-F.

26. The oligonucleotide of claim 24, wherein the first nucleotide at the 5'-end of the oligonucleotide has the structure:

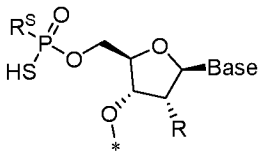


, or a salt thereof.

27. The oligonucleotide of claim 24, wherein the first nucleotide at the 5'-end of the oligonucleotide has the structure:



or



, or a salt thereof.

28. The oligonucleotide of any one of claims 25-27, wherein Base is uridine.

29. The oligonucleotide of any one of claims 25-28, wherein R is methoxy or hydrogen.

30. The oligonucleotide of claim 17, wherein the oligonucleotide contains at least one cyclic disulfide moiety at the 3'-end of the oligonucleotide.

31. The oligonucleotide of claim 17, wherein the oligonucleotide contains at least one cyclic disulfide moiety at an internal position of the oligonucleotide.

32. The oligonucleotide of claim 17, wherein the oligonucleotide is a single-stranded oligonucleotide.

33. The oligonucleotide of claim 17, wherein the oligonucleotide is a double-stranded oligonucleotide comprising a sense strand and an antisense strand.

34. The oligonucleotide of claim 33, wherein the oligonucleotide contains at least one cyclic disulfide moiety at the antisense strand, sense strand, or both strands of the oligonucleotide.

35. The oligonucleotide of claim 34, wherein the sense strand is 21 nucleotides in length, and the antisense strand is 23 nucleotides in length, wherein the strands form a double-stranded region of 21 consecutive base pairs having a 2-nucleotide long single-stranded overhangs at the 3'-end.

36. The oligonucleotide of claim 33, wherein the oligonucleotide contains at least one cyclic disulfide moiety at the 5'-end of the antisense strand and at least one targeting ligand at the

3’-end of the sense strand.

37. The oligonucleotide of claim 17, wherein the oligonucleotide contains one or more 2’-O modifications selected from the group consisting of 2’-deoxy, 2’-O-methoxyalkyl, 2’-O-methyl, 2’-O-allyl, 2’-C-allyl, 2’-fluoro, 2’-O-N-methylacetamido (2'-O-NMA), 2’-O-dimethylaminoethoxyethyl (2’-O-DMAEOE), 2'-O-aminopropyl (2'-O-AP), and 2’-ara-F.

38. The oligonucleotide of claim 33, wherein the sense and the antisense strands comprise no more than ten 2’-fluoro modified nucleotides.

39. The oligonucleotide of claim 33, wherein the sense and antisense strands comprise at least 50%, at least 60%, or least 70% of 2’-OMe modified nucleotides.

40. The oligonucleotide of claim 33, wherein the sense strand or antisense strand comprises at least two phosphorothioate linkages at the 5’-end or at the 3’-end.

41. A pharmaceutical composition comprising the oligonucleotide of claim 17, and a pharmaceutically acceptable excipient.

42. A method for reducing or inhibiting the expression of a target gene in a subject, comprising:

administering to the subject the oligonucleotide of claim 17, in an amount sufficient to inhibit expression of the target gene.

43. A method for modifying an oligonucleotide comprising:

contacting the oligonucleotide with the compound according to claim 1 under conditions suitable for reacting the compound with the oligonucleotide, wherein the oligonucleotide comprises a free hydroxyl group.

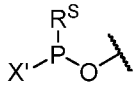
44. The method of claim 43, wherein the free hydroxyl group is part of the 5’-terminal or part of the 3’-terminal nucleotide.

45. The method of claim 43, wherein the oligonucleotide comprises a 5’-OH group or 3’-OH

group.

46. A method for preparing a modified oligonucleotide, comprising:

oxidizing a first oligonucleotide comprising a group of formula (A):



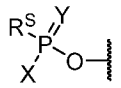
(A)

or a salt thereof, wherein:

R^S is a cyclic disulfide moiety;

X' is $-OR^{13}$ or $-SR^{13}$, wherein R^{13} is alkyl, alkenyl, alkynyl, cycloalkyl, aryl, aralkyl, ω-amino alkyl, ω-hydroxy alkyl, ω-hydroxy alkenyl, alkylcarbonyl, or arylcarbonyl, each of which can be optionally substituted with one or more R^{sub} groups;

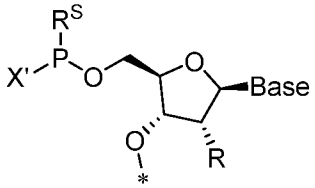
under conditions suitable for forming a modified oligonucleotide comprising a group of formula (B):



(B)

or a salt thereof, wherein Y is O or S; and X is -OH, -SH or X'.

47. The method of claim 46, wherein the first nucleotide at the 5'-end of the first oligonucleotide is according to formula (C):



(C)

or a salt thereof, wherein:

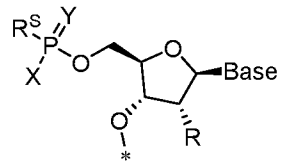
* represents a bond to the subsequent optionally modified internucleotide linkage;

Base is an optionally modified nucleobase; and

R is H, OH, O-methoxyalkyl, O-methyl, O-allyl, CH_2-allyl, fluoro, O-N-methylacetamido (O-NMA), O-dimethylaminoethoxyethyl (O-DMAEOE), O-aminopropyl (O-AP), or ara-F, and

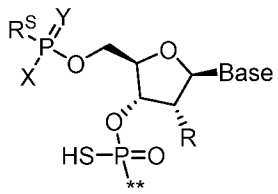
the first nucleotide at the 5'-end of the modified oligonucleotide has the structure of

formula (D):

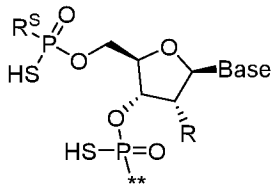


(D).

48. The method of claim 46, wherein the first nucleotide at the 5'-end of the modified oligonucleotide has the structure of formula (E) or (F):



(E),



(F),

or a salt thereof, wherein ** represent the bond to the subsequent nucleotide.

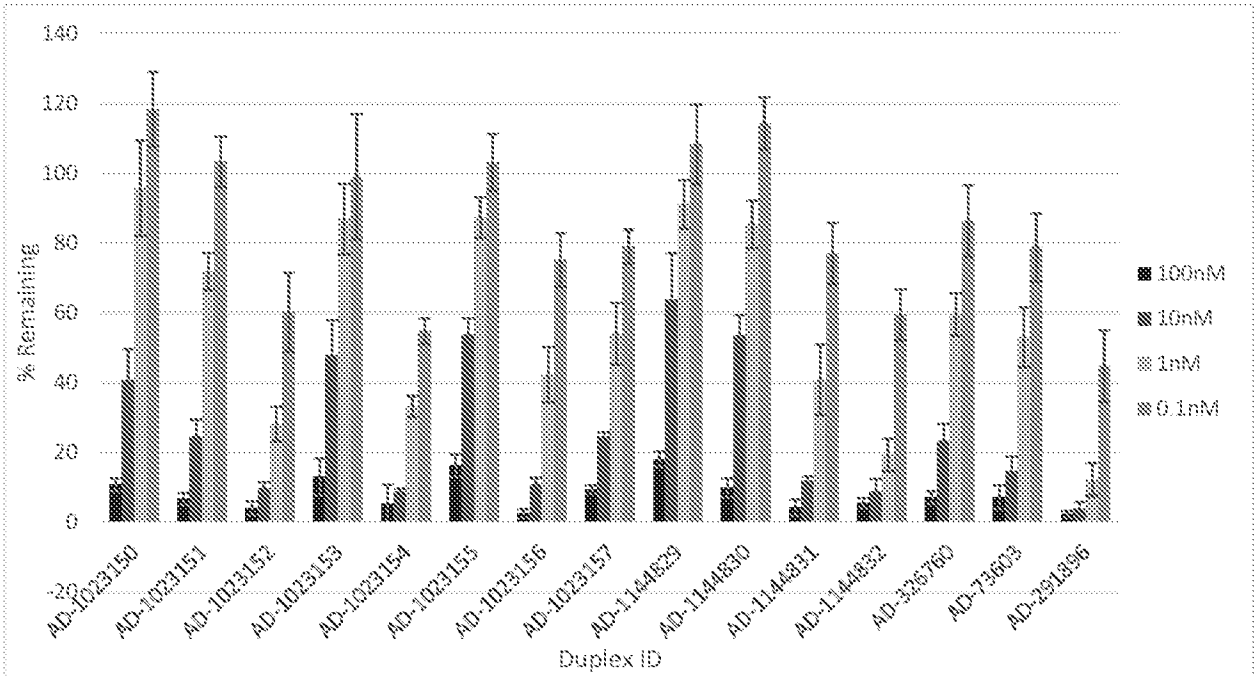


Figure 1

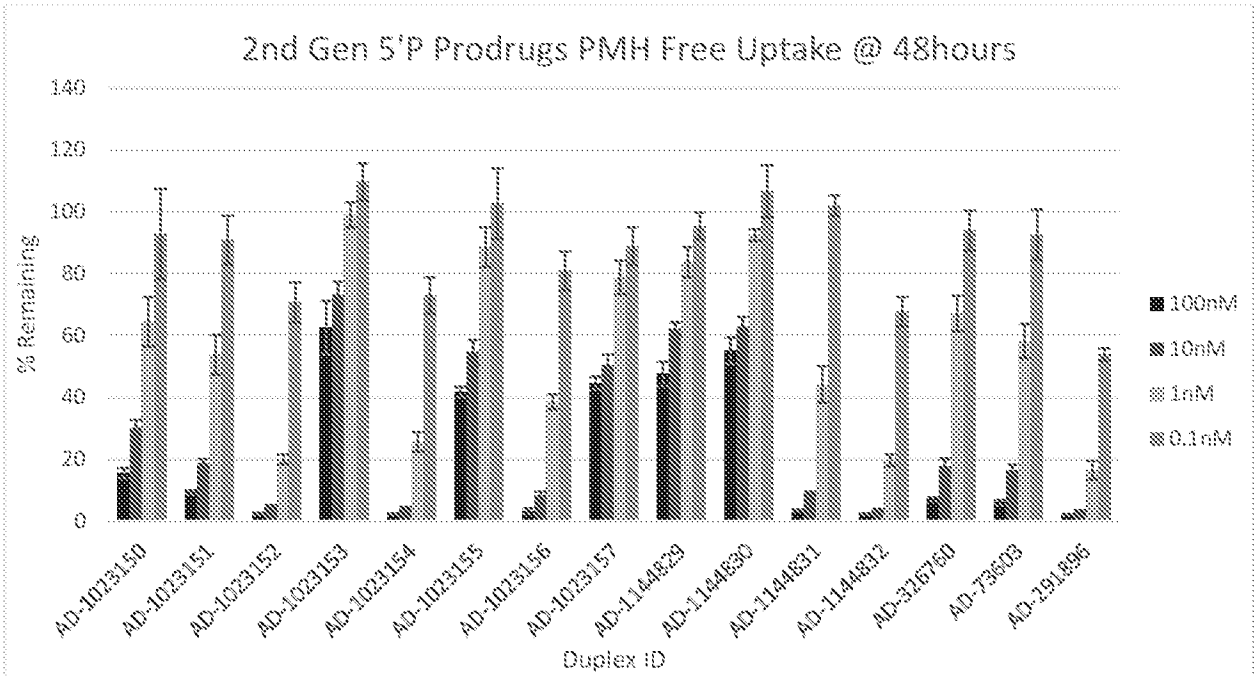


Figure 2

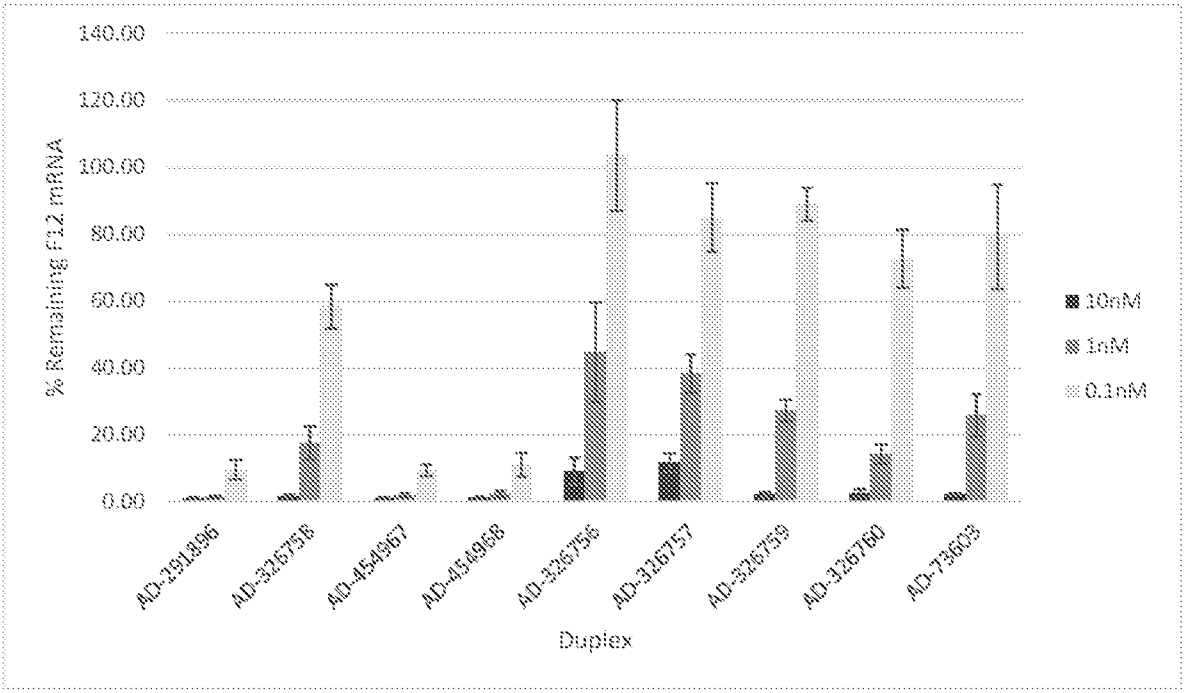
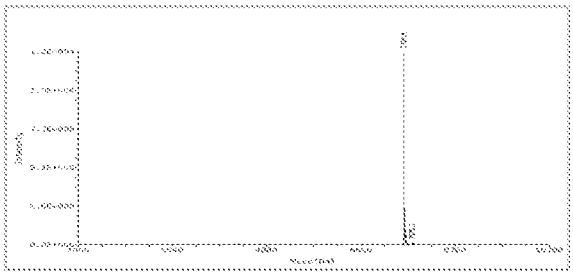

140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
% Remaining F12 mRNA
10nM
1nM
0.1nM
AD-191896
AD-326758
AD-454967
AD-454968
AD-326756
AD-326757
AD-326759
AD-326760
AD-73603
Duplex

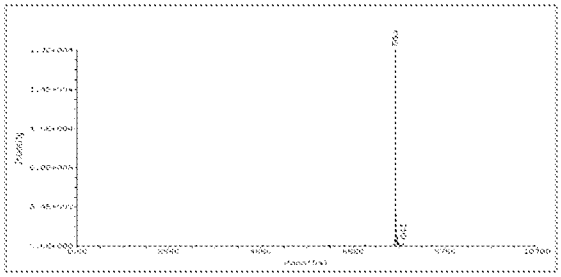
Figure 3

A. A-515489

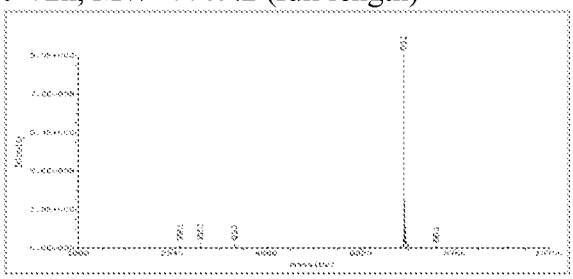
t=0h, MW=7709.1 (full length)



t=24h, MW=7709.0

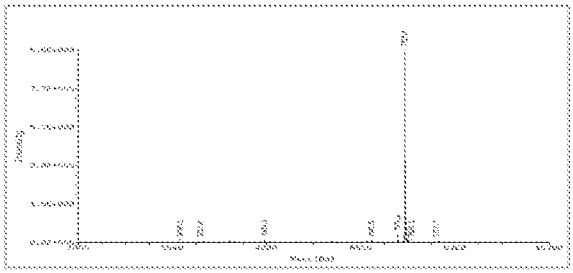


t=72h, MW=7709.2 (full length)

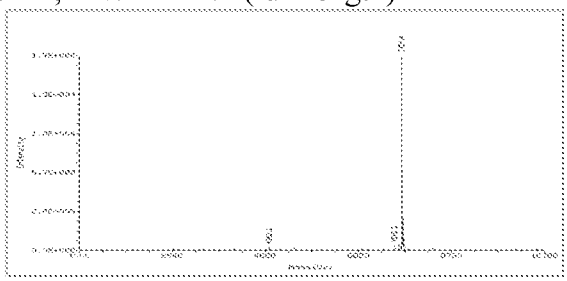


B. A-515491

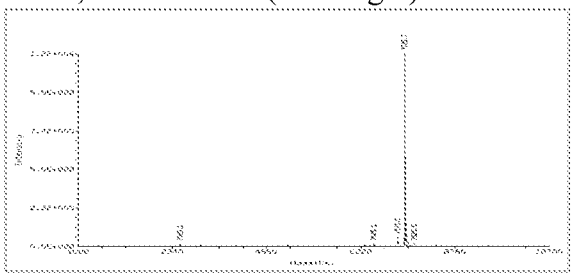
t=0h, MW=7725.4 (full length)



t=24, MW=7725.4 (full length)

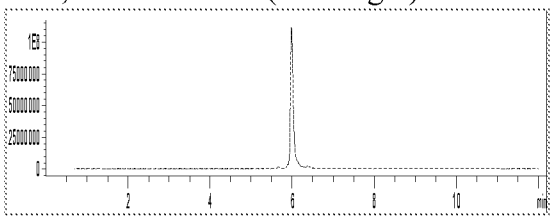


t=72h, MW=7725.7 (full length)

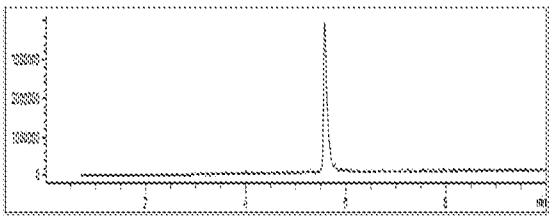


C. A-1875173

t=0h, MW=7721.32 (full length)

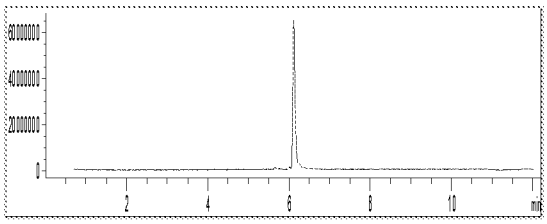


t=24h, MW=7721.09 (full length)

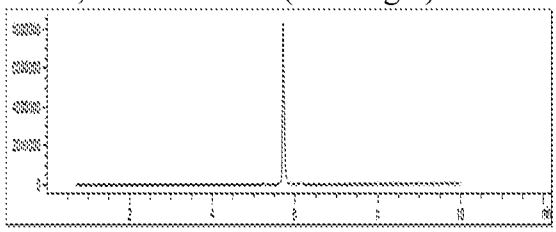


D. A-1875172

t=0h, MW=7705.14 (full length)

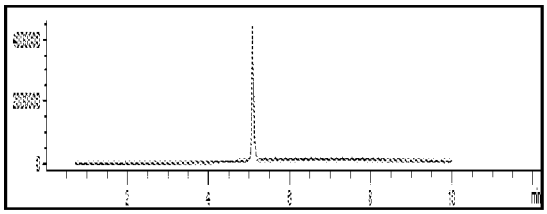


t=24h, MW=7705.11 (full length)

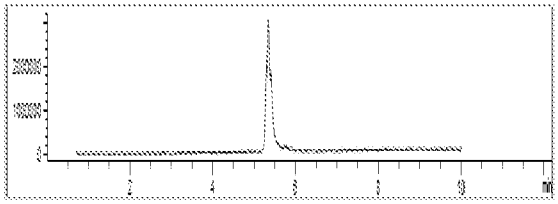


E. A-1875175

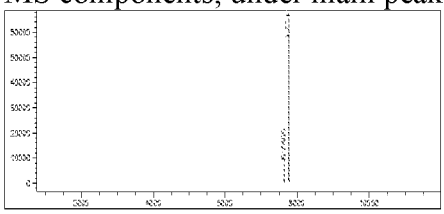
T=0h, MW=7744.6 (full length)



t=24h, MW=7744.4 (full length); under main peak see MW 7608.67 (5′PS)

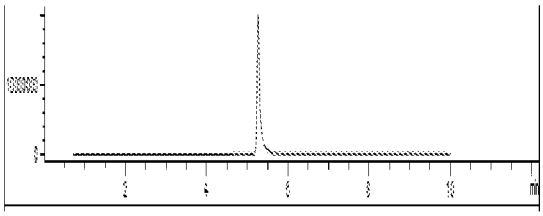


MS components; under main peak at 24h

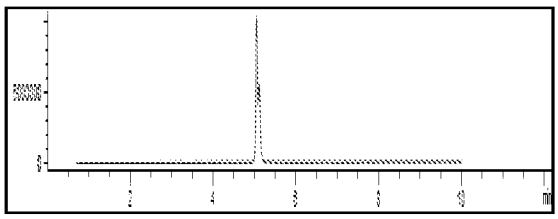


F. A-1875184

t=0h, MW=7728.37 (full length)

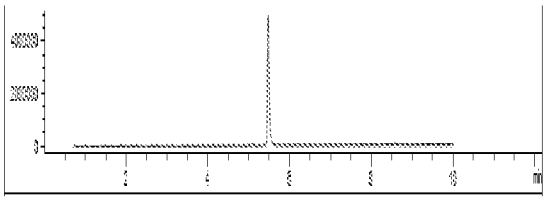


t=24h, MW=7728.37 (full length)

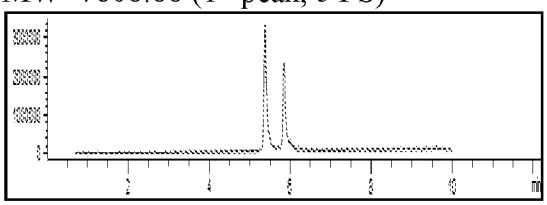


G. A-1875179

t=0h, MW=7765.94 (full length)

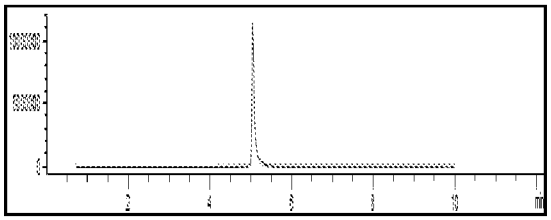


t=24h, MW=7755.03 (2nd peak, full length), MW=7608.88 (1st peak, 5′PS)

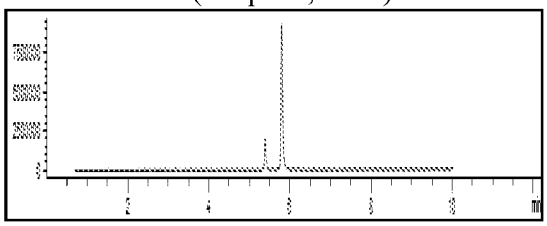


H. A-1875178

t=0h, MW=7739.01 (full length)

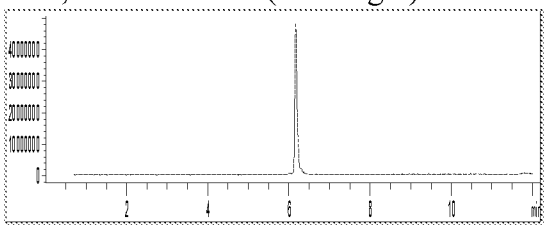


t=24h, MW=7739.94 (2nd peak, full length), MW=7592.83 (1st peak, 5′PS)

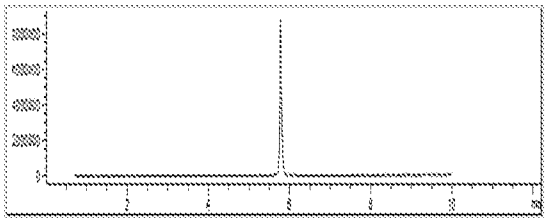


I. A-1875180

t=0h, MW=7753.11 (full length)

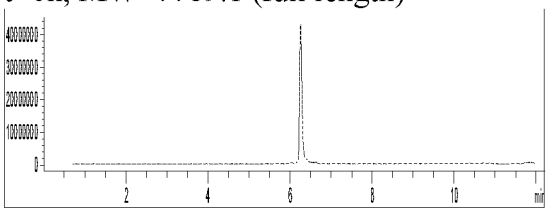


t=24h, MW=7753.16 (full length)



J. A-1875181

t=0h, MW=7769.1 (full length)



t=24h, MW=7769.02 (full length)

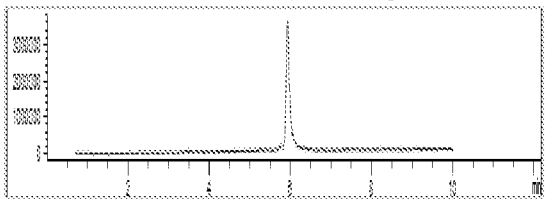


Figure 4

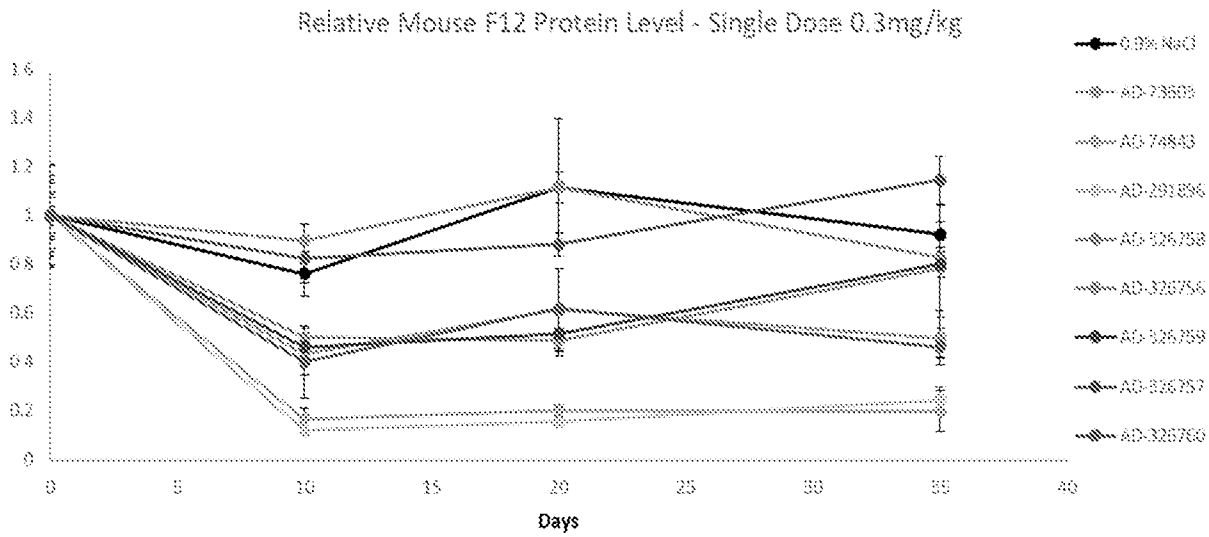


Figure 5

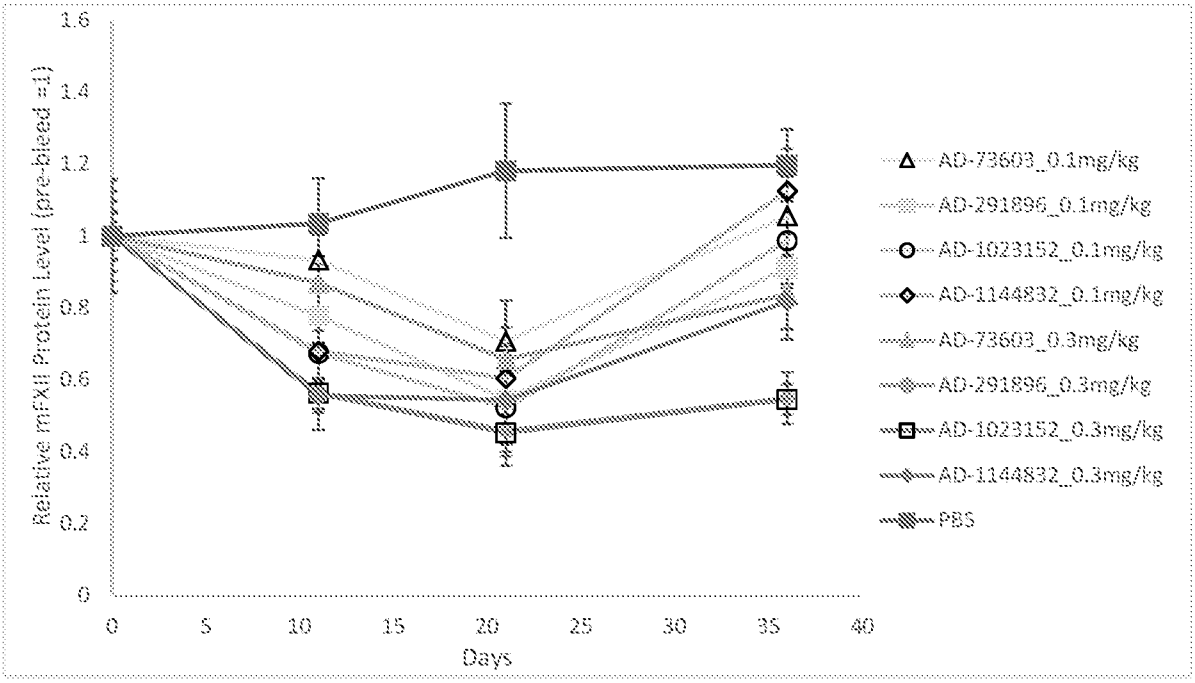


Figure 6

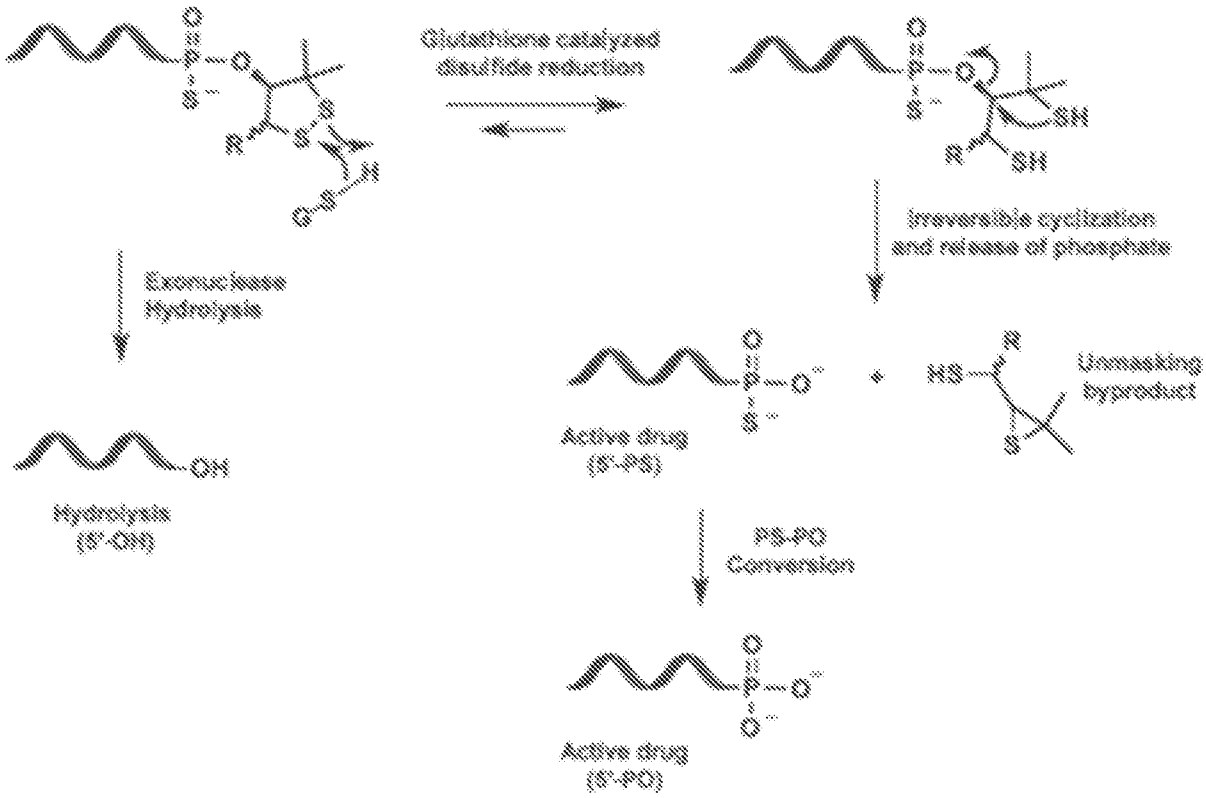


Figure 7

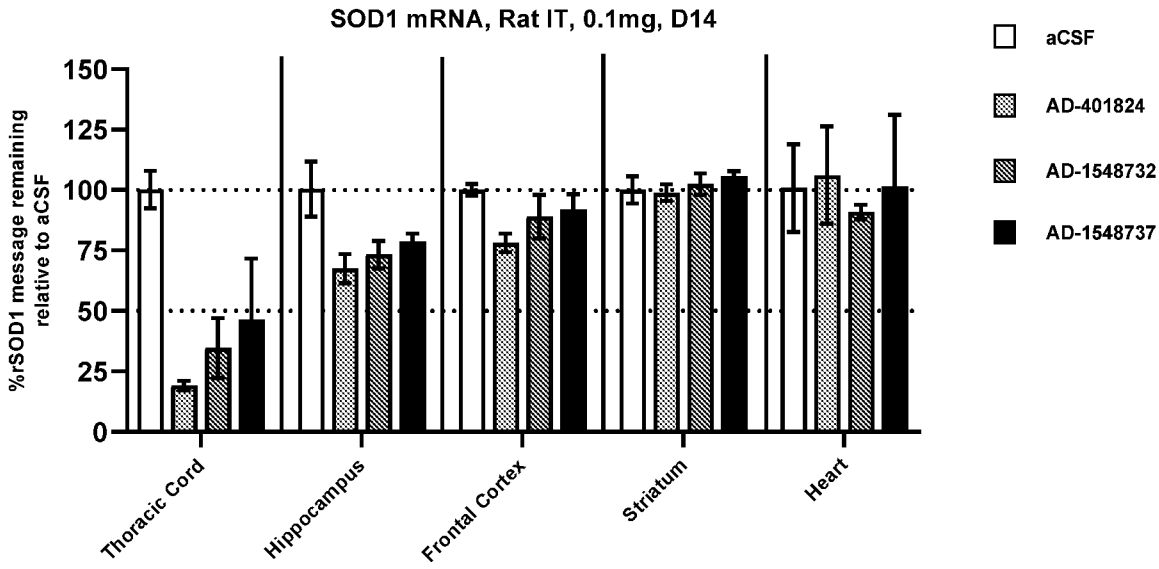


Figure 8

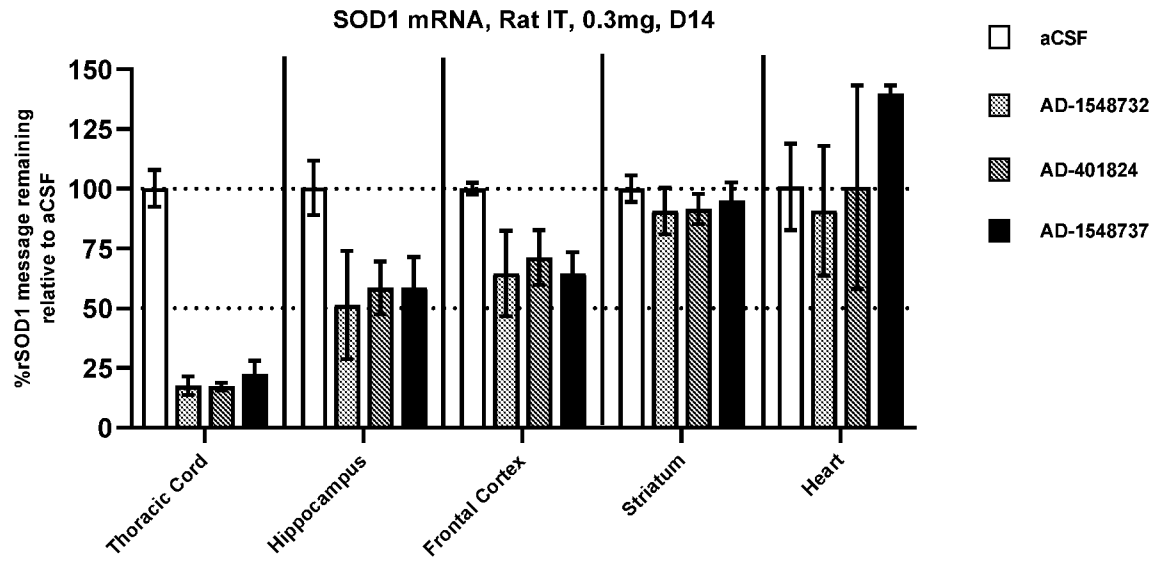


Figure 9

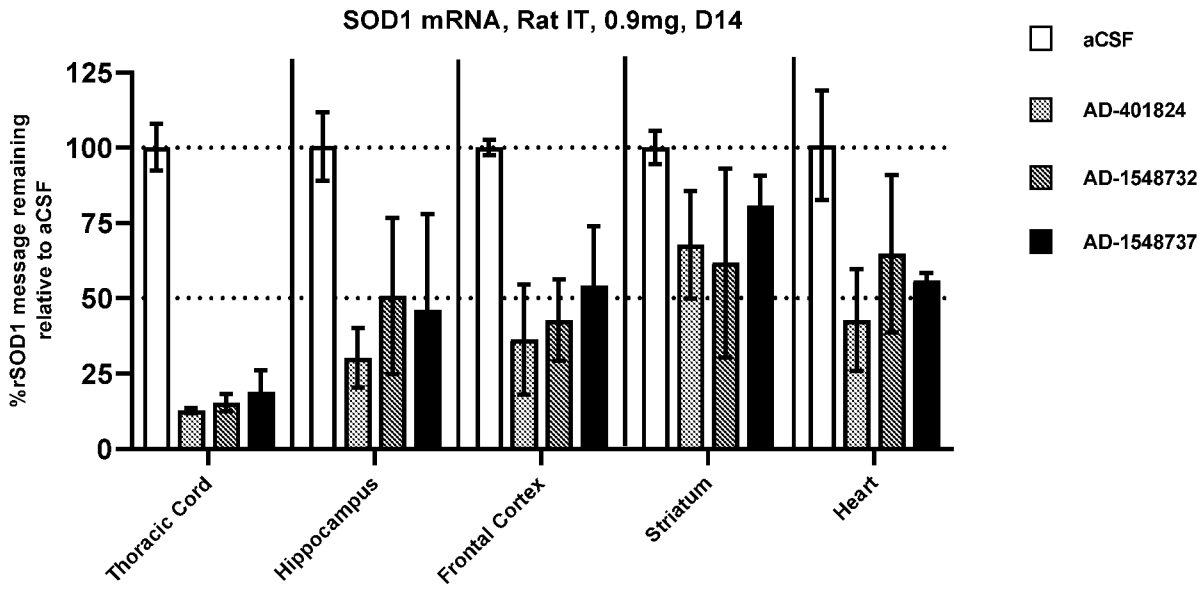


Figure 10

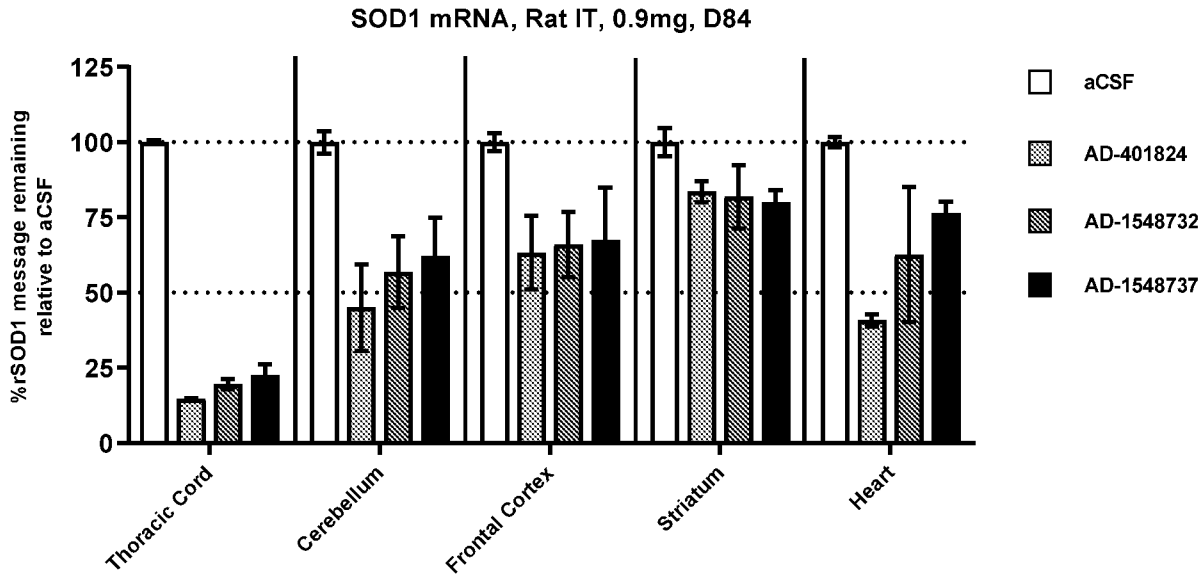


Figure 11

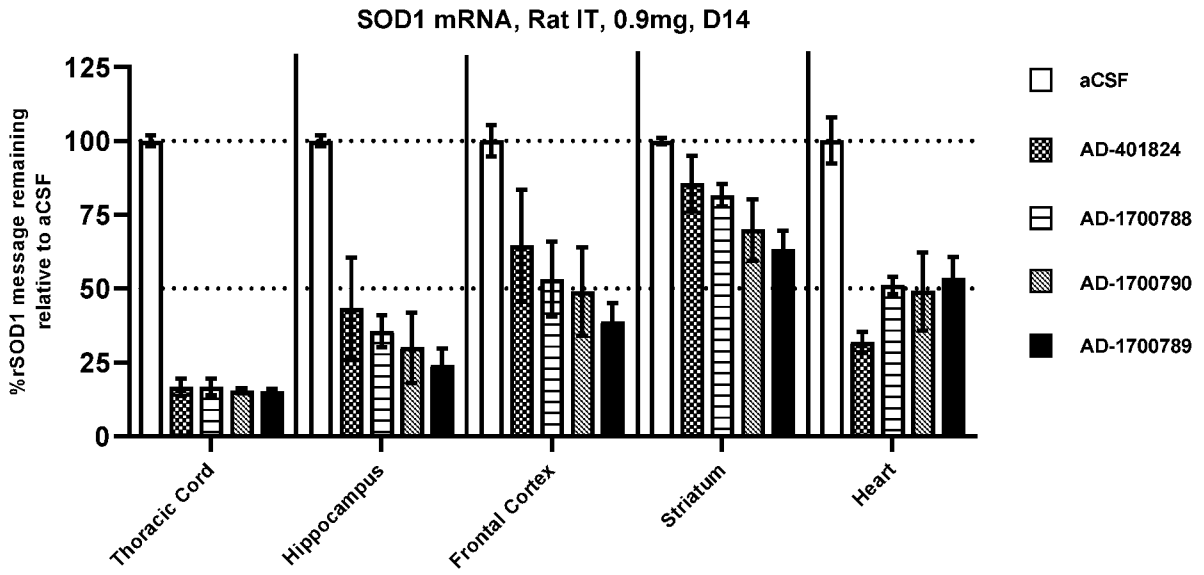


Figure 12

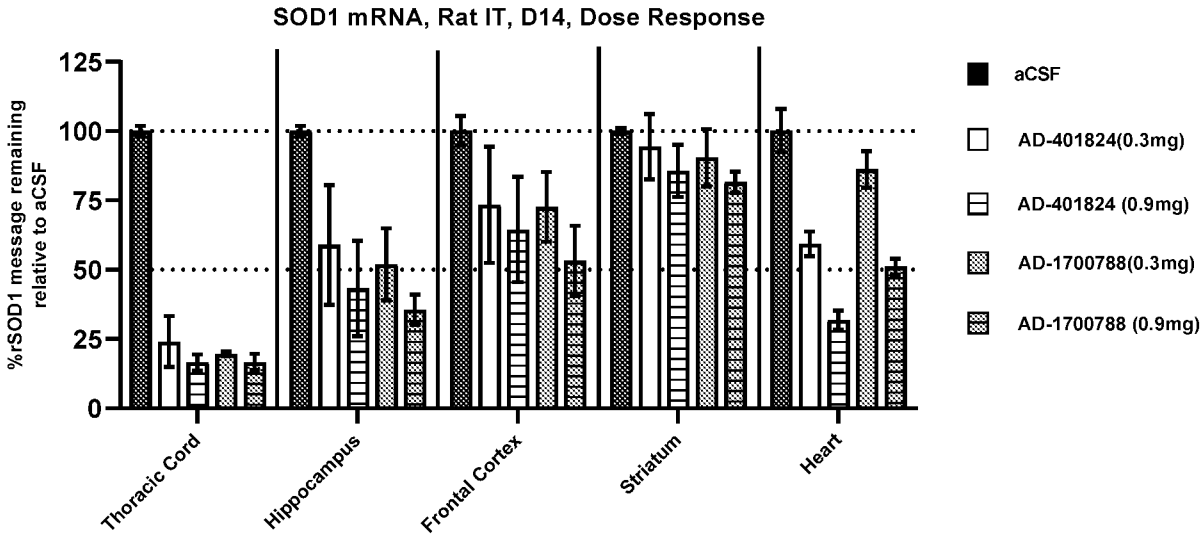


Figure 13

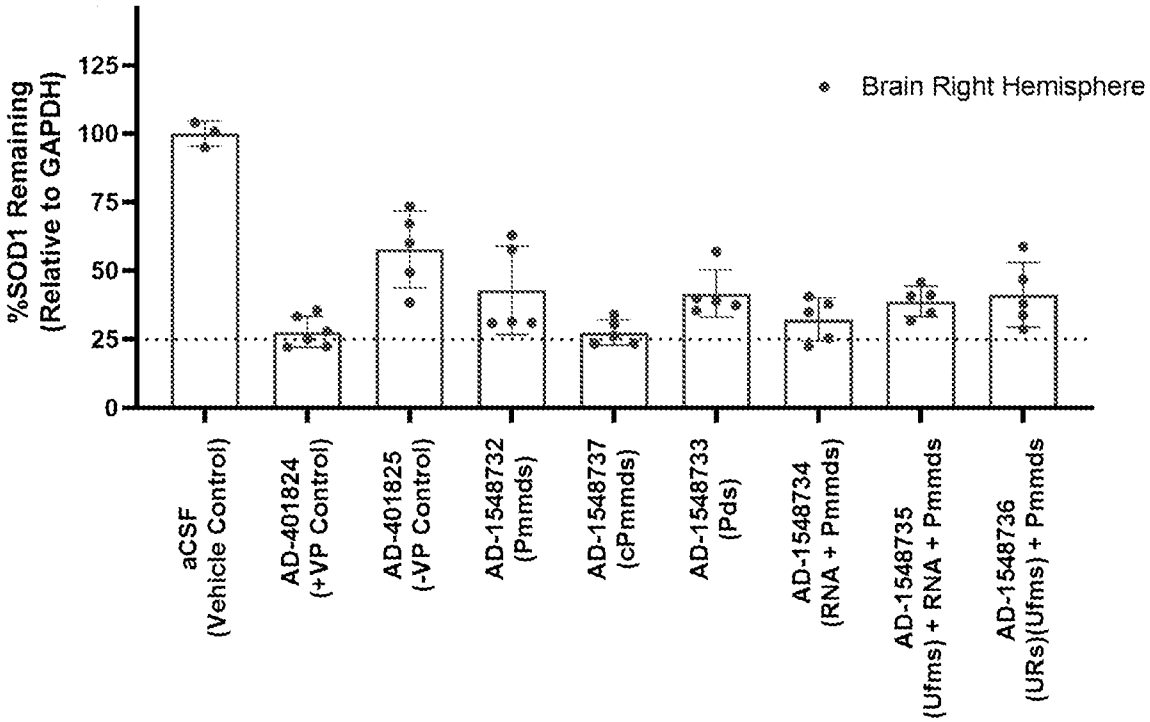

%SOD1 Remaining
(Relative to GAPDH)
125
100
75
50
25
0
Brain Right Hemisphere
aCSF
(Vehicle Control)
AD-401824
(+VP Control)
AD-401825
(-VP Control)
AD-1548732
(Pmmds)
AD-1548737
(cPmmds)
AD-1548733
(Pds)
AD-1548734
(RNA + Pmmds)
AD-1548735
(Ufms) + RNA + Pmmds
AD-1548736
(URs)(Ufms) + Pmmds

Figure 14