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(54) **Title**: CHELATED COPPER FOR USE IN THE PREPARATION OF CONJUGATED OLIGONUCLEOTIDES

(57) **Abstract**: In one embodiment, the invention features a process for preparing ligand conjugated oligonucleotides comprising a step of contacting a nucleoside or oligonucleotide containing an alkyne moiety with ligand containing an azide moiety in the presence of a solid supported copper catalyst of formula A, B, C or D.

Chelated Copper for use in the Preparation of Conjugated Oligonucleotides

PRIORITY CLAIM

This application claims priority to U.S. Provisional Application No. 61/299,302, filed January 28, 2010.

FIELD OF INVENTION

The present invention relates to the field of conjugation of ligands to oligonucleotides with "click" chemistry in the presence of solid supported Cu(I) catalyst.

BACKGROUND

Oligonucleotide compounds have important therapeutic applications in medicine. Oligonucleotides can be used to silence genes that are responsible for a particular disease. Gene-silencing prevents formation of a protein by inhibiting translation. Importantly, gene-silencing agents are a promising alternative to traditional small, organic compounds that inhibit the function of the protein linked to the disease. siRNA, antisense RNA, and micro-RNA are oligonucleotides that prevent the formation of proteins by gene-silencing.

The opportunity to use of nucleic acid based therapies holds significant promise, providing solutions to medical problems that could not be addressed with current, traditional medicines. The location and sequences of an increasing number of disease-related genes are being identified, and clinical testing of nucleic acid-based therapeutics for a variety of diseases is now underway.

Despite the different synthetic strategies developed for conjugation of various ligands to the oligonucleotides, the synthesis of ligand-oligonucleotide conjugates is anything but trivial and requires extensive expertise in organic chemistry and solid-phase synthesis. A real advance would be to use a coupling reaction that can be utilized for a large variety of ligands and oligonucleotides. The Huisgen 1,3-dipolar cycloaddition of alkynes and azides, the "click" reaction, is especially attractive for irreversible coupling of two molecules under mild conditions. The "click" chemistry has recently emerged as

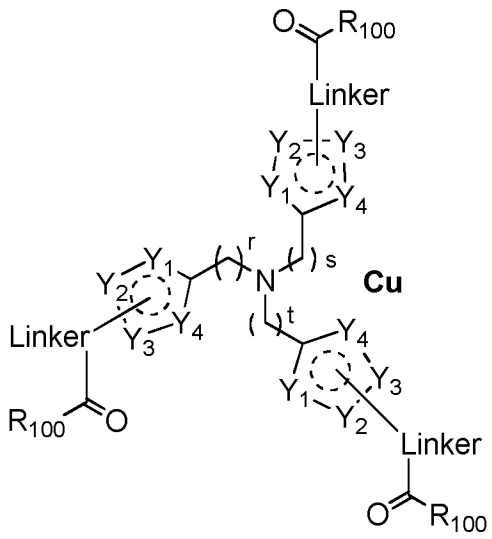
an efficient strategy to conjugate carbohydrates, peptides and proteins, fluorescent labels and lipids to oligonucleotides. Therefore, there is a clear need for new reagents that can be utilize for "click" chemistry for conjugation of ligands to oligonucleotides. The present invention is directed to this very important end.

SUMMARY

The invention relates to chelated copper on solid support enables copper free click conjugation of ligands and drug carriers (nano particles, polymers and other drug carrier system) to nucleic acids (siRNA, ssRNA, microRNAs, antisense, aptamer, decoy etc.). The chelated immobilized copper on solid support catalyzes click reaction of completely deprotected oligonucleotides with ligands of choice; and it makes the medium free of floating copper. Conventional click conjugation on completely deprotected oligonucleotides (RNA or DNA) with functionalized ligands are challenging due to free floating Cu ion which ion-pair/coordinate with the oligonucleotide. The separation of copper completely from the product during purification is extremely difficult after conjugation by using conventional click reagent due to chelation or ion-pairing of free copper with the nucleic acid. In addition, the immobilized copper catalysts described herein are very efficient and recyclable.

DETAILED DESCRIPTION

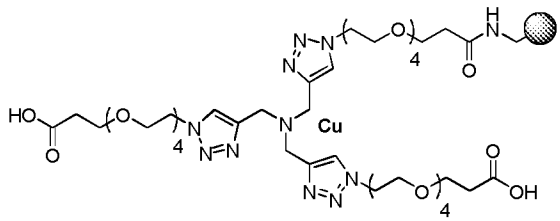
In one embodiment, the chelated copper on solid support of the present invention features a compound of formula A:



(A)

wherein Y_1-Y_4 are each independently CR^P, CR^P_2, N, NR^N, O, and S; r, s and t are each indenpendently 1-6; R_{100} is independently for each occurrence OH, OR^P, or solid support; where R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl; R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; each linker can be the same or different; and provided that at least one R_{100} is bound to a solid support.

Representative chelated copper of formula (A) is selected from the group consisting of:



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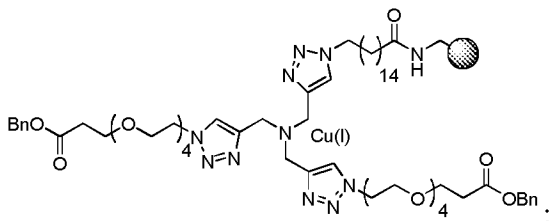

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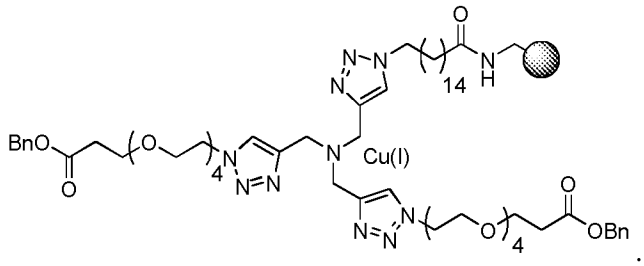
and



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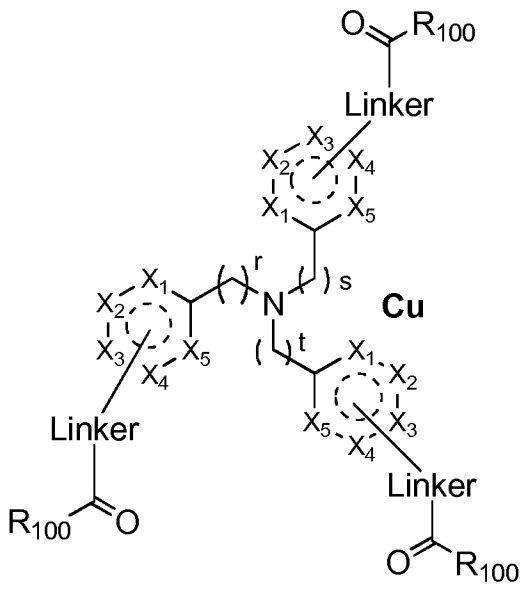
In one embodiment, the chelated copper on solid support of the present invention features a compound of formula B:

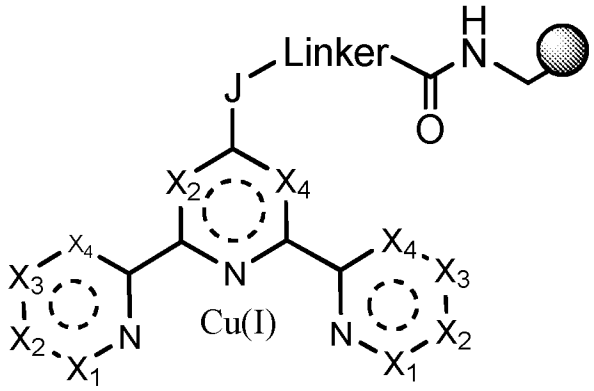


(B)

wherein X_1-X_6 are each independently CR^P, CR^P_2, N, NR^N, O, and S; r, s and t are each indenpendently 1-6; R_{100} is independently for each occurrence OH, OR^P, or solid support; where R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl; R^N is independently

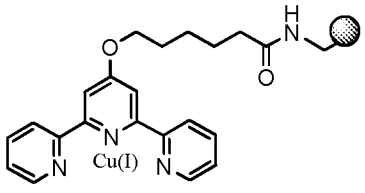
for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; each linker can be the same or different; and provided that at least one R_{100} is bound to a solid support.

In one embodiment, the chelated copper on solid support of the present invention features a compound of formula C:



(C)

wherein X_1-X_4 are each independently CR^P, CR^P_2, N, NR^N, O, and S; where R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl; R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; and  is a solid support.

A representative chelated copper of formula (C) is 

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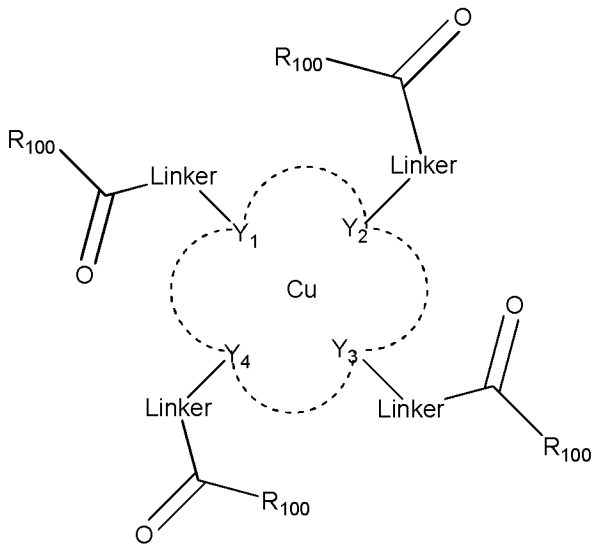
In one embodiment, the chelated copper on solid support of the present invention features a compound of formula D:



(D)

wherein ---------- is a bond or absent; Y_1-Y_4 are each independently N, NR^N, O, and S; where R_{100} is independently for each occurrence OH, OR^P, or solid support; R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; each linker can be the same or different; each linker can be the same or different; and provided that at least one R_{100} is bound to a solid support.

The term "solid support" as used herein refers to a flexible or non-flexible support that is suitable for carrying said immobilized chelated copper. Said solid support may be homogenous or inhomogeneous. For example, said solid support may consist of different materials having the same or different properties with respect to flexibility and immobilization, for instance, or said solid support may consist of one material exhibiting a plurality of properties also comprising flexibility and immobilization properties. Such supports are well known in the art and comprise, inter alia, commercially available column materials, polystyrene beads, latex beads, magnetic beads, colloid metal particles, glass and/or silicon chips and surfaces, nitrocellulose strips, membranes, sheets,

duracytes, wells and walls of reaction trays, plastic tubes etc. Examples of well-known carriers include glass, polystyrene, polyvinyl chloride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. Said solid support may comprise glass-, polypropylene- or silicon-chips, membranes oligonucleotide-conjugated beads or bead arrays. Supports used in solid phase synthesis are typically substantially inert and nonreactive with the solid phase synthesis reagents. Methods of using solid supports in solid phase synthesis are well known in the art and may include, but are not limited to, those described in U.S. Pat. Nos. 4,415,732, 4,458,066; 4,500,707, 4,668,777; 4,973,679, and 5,132,418 issued to Caruthers, and U.S. Pat. No. 4,725,677 and Re. 34,069 issued to Koster, each of which are herein incorporated by reference in their entirety for all purposes.

In one embodiment, the solid support is selected from polystyrenes with different degree of cross linking, glass support (controlled pore glass) or silica support, PEGylated polystyrene, other swellable or non-swellable polymer supports for e.g, NittoPhase from NittoDenko and the like, including liquid polymers such as PEGs and Ionic liquids.

In one embodiment, the chelated copper catalyst is immobilized on soluble or insoluble solid support so that the product is free of any copper catalyst.

In one embodiment, the invention features a process for preparing a ligand conjugated oligonucleotide comprising a step of contacting a nucleoside or oligonucleotide containing an alkyne moiety with ligand containing an azide moiety in the presence of a solid supported copper catalyst.

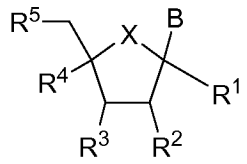
In one embodiment, the invention features a process for preparing a ligand conjugated oligonucleotide comprising a step of contacting a nucleoside or oligonucleotide containing an alkyne moiety with ligand containing an azide moiety in the presence of a solid supported copper catalyst of formula (A), (B), (C), or (D).

In one embodiment, the invention features a process for preparing a ligand conjugated oligonucleotide comprising a step of contacting a nucleoside or

oligonucleotide containing an azide moiety with ligand containing an alkyne moiety in the presence of a solid supported copper catalyst of formula (A), (B), (C), or (D).

In one embodiment, the invention features a process for preparing a ligand conjugated oligonucleotide comprising a step of contacting a nucleoside or oligonucleotide containing an azide moiety with ligand containing an alkyne moiety in the presence of a solid supported copper catalyst.

In one aspect, the invention features a method for preparing compound having the structure shown in formula (I) by using a chelated copper catalyst of formula (A), (B), (C), or (D);



Formula (I)

wherein:

X is O, S, NR^N or CR^P_2;

B is independently for each occurrence hydrogen, optionally substituted natural or non-natural nucleobase, optionally substituted triazole or optionally substituted tetrazole; NH-C(O)-O-$C(CH_2B_1)_3$, NH-C(O)-NH-$C(CH_2B_1)_3$; where B_1 is halogen, mesylate, N_3, CN, optionally substituted triazole or optionally substituted tetrazole;

R^1, R^2, R^3, R^4 and R^5 are each independently for each occurrence H, OR^6, F, $N(R^N)_2$, N_3, CN, -J-linker-N_3, -J-linker-CN, -J-linker-C≡R^8, -J-linker-cycloalkyne, -J-linker-R_L, or -J-linker-Q-linker-R^L;

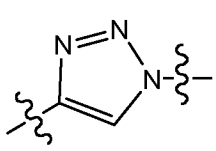
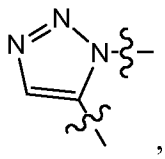
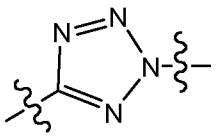
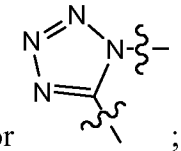
J is absent, O, S, NR^N, OC(O)NH, NHC(O)O, C(O)NH, NHC(O), NHSO, $NHSO_2$, $NHSO_2NH$, OC(O), C(O)O, OC(O)O, NHC(O)NH, NHC(S)NH, OC(S)NH, $OP(N(R^P)_2)O$, or $OP(N(R^P)_2)$;

R^6 is independently for each occurrence hydrogen, hydroxyl protecting group, optionally substituted alkyl, optionally substituted aryl, optionally substituted cycloalkyl,

optionally substituted aralkyl, optionally substituted alkenyl, optionally substituted heteroaryl, polyethyleneglycol (PEG), a phosphate, a diphosphate, a triphosphate, a phosphonate, a phosphonothioate, a phosphonodithioate, a phosphorothioate, a phosphorothiolate, a phosphorodithioate, a phosphorothiolothionate, a phosphodiester, a phosphotriester, an activated phosphate group, an activated phosphite group, a phosphoramidite, a solid support, -P(Z^1)(Z^2)-O-nucleoside, -P(Z^1)(Z^2)-O-oligonucleotide, -P(Z^1)(Z^2)-formula (I), -P(Z^1)(O-linker-Q-linker-R^L)-O-nucleoside, P(Z^1)(O- linker-R^L)-O-nucleoside, -P(Z^1)(O-linker-N_3)-O-nucleoside, P(Z^1)(O-linker-CN)-O-nucleoside, P(Z^1)(O-linker-C≡R^8)-O-nucleoside, P(Z^1)(O-linker-cycloalkyne)-O-nucleoside, -P(Z^1)(O-linker-Q-linker-R^L)-O-oligonucleotide, P(Z^1)(O-linker-R^L)-O-oligonucleotide, P(Z^1)(O-linker-N_3)-O-oligonucleotide, -P(Z^1)(O-linker-CN)-O-oligonucleotide, P(Z^1)(O-linker-C≡R^8)-O-oligonucleotide, P(Z^1)(O-linker-cycloalkyne)-O-oligonucleotide, -P(Z^1)(-linker-Q-linker-R^L)-O-nucleoside, -P(Z^1)(-linker-Q-R^L)-O-nucleoside, -P(Z^1)(-linker-N_3)-O-nucleoside, P(Z^1)(-linker-CN)-O-nucleoside, P(Z^1)(-linker-C≡R^8)-O-nucleoside, P(Z^1)(-linker-cycloalkyne)-O-nucleoside, -P(Z^1)(-linker-Q-linker-R^L)-O-oligonucleotide, -P(Z^1)(-linker-R^L)-O-oligonucleotide, P(Z^1)(-linker-N_3)-O-oligonucleotide, -P(Z^1)(-linker-CN)-O-oligonucleotide, P(Z^1)(-linker-C≡R^8)-O-oligonucleotide or P(Z^1)(-linker-cycloalkyne)-O-oligonucleotide;

R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group;

R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl;

Q is absent or independently for each occurrence  ,  ,  , or  ;

R^L is hydrogen or a ligand;

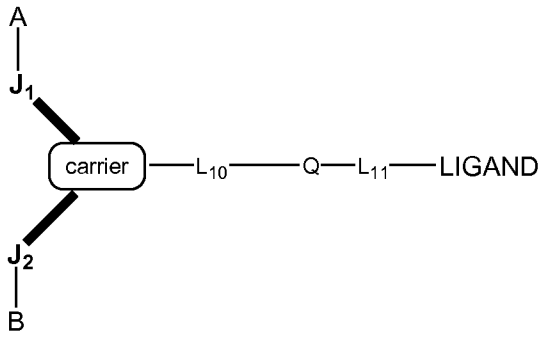
R^8 is N or CR^9;

R^9 is H, optionally substituted alkyl or silyl;

Z^1 and Z^2 are each independently for each occurrence O, S or optionally substituted alkyl;

provided that at least one of R^1, R^2, R^3, R^4 and R^5 is -J-Linker-Q-Linker-R^L or -Linker-Q-R^L when B is an unsubstitued natural base.

In one embodiment, the invention features a method for preparing a compound having the structure shown in formula (II) by using a chelated copper catalyst of formula (A), (B), (C), or (D);



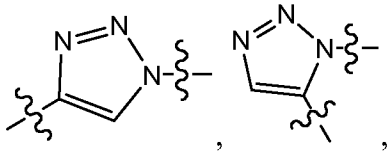
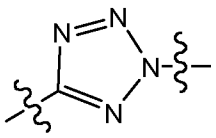
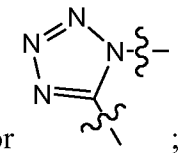
(II)

A and B are independently for each occurrence hydrogen, protecting group, optionally substituted aliphatic, optionally substituted aryl, optionally substituted heteroaryl, polyethyleneglycol (PEG), a phosphate, a diphosphate, a triphosphate, a phosphonate, a phosphonothioate, a phosphonodithioate, a phosphorothioate, a phosphorothiolate, a phosphorodithioate, a phosphorothiolothionate, a phosphodiester, a phosphotriester, an activated phosphate group, an activated phosphite group, a

phosphoramidite, a solid support, -P(Z^1)(Z^2)-O-nucleoside, or -P(Z^1)(Z^2)-O-oligonucleotide; wherein Z^1 and Z^2 are each independently for each occurrence O, S or optionally substituted alkyl;

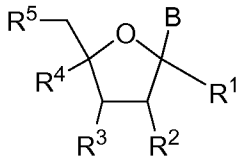
J_1 and J_2 are independently O, S, NR^N, optionally substituted alkyl, OC(O)NH, NHC(O)O, C(O)NH, NHC(O), OC(O), C(O)O, OC(O)O, NHC(O)NH, NHC(S)NH, OC(S)NH, OP(N(R^P)$_2$)O, or OP(N(R^P)$_2$);

carrier is cyclic group or acyclic group; preferably, the cyclic group is selected from pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolane, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuryl and and decalin; preferably, the acyclic group is selected from serinol backbone or diethanolamine backbone;

Q is independently for each occurrence  , ,  , or  ; and

L_{10} and L_{11} are independently absent or a linker.

In one embodiment, the invention features a method for preparing a compound having the structure shown in formula (III) by using a chelated copper catalyst of formula (A), (B), (C), or (D);



Formula (III)

In one embodiment, the R^2 and R^4 of the same compound are connected together to form a "locked" compound similar to a locked nucleic acid (LNA).

In one embodiment, R^1 and R^4 are H.

In one embodiment, R^1 is -O-Linker-Q-Linker-R^L, -OC(O)N(R^N)-Linker-Q-Linker-R^L or -Linker-Q-Linker-R^L, B is H.

In one embodiment, the R^2 and R^4 of the same compound are connected together to form a "locked" compound similar to a locked nucleic acid (LNA).

In one embodiment, when B is hydrogen, R^1 is -O-Linker-Q-Linker-R^L, -OC(O)N(R^7)-Linker-Q-Linker-R^L or -Linker-Q-Linker-R^L.

In one embodiment, B is H.

In one embodiment, B is pyrimidine substituted at C5 position.

In one embodiment, R^2 is OR^6 and R^3 is -O-Linker-Q-Linker-R^L, -OC(O)N(R^N)-Linker-Q-Linker-R^L or -Linker-Q-Linker-R^L and R^L is present.

In one embodiment, R^3 is OR^6 and R^2 is -O-Linker-Q-Linker-R^L, -OC(O)N(R^N)-Linker-Q-Linker-R^L or -Linker-Q-Linker-R^L and R^L is present.

In one embodiment, R^2 is OH.

In one embodiment, R^9 is H.

In one embodiment, R^5 is -O-Linker-Q-Linker-R^L, -OC(O)N(R^N)-Linker-Q-Linker-R^L or -Linker-Q-Linker-R^L and R^L is present.

In one embodiment, R^5 is $-OC(O)NH(CH_2)_fC{\equiv}CR^9$, and f is 1-20.

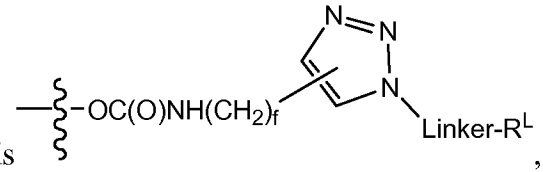
In one embodiment, R^4 is $-OC(O)NH(CH_2)_fC{\equiv}CR^9$, and f is 1-20.

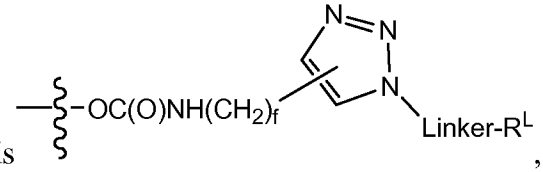
In one embodiment, R^3 is $-OC(O)NH(CH_2)_fC{\equiv}CR^9$, and f is 1-20.

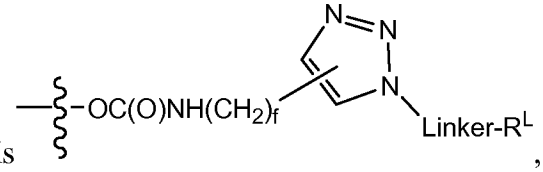
In one embodiment, R^2 is $-OC(O)NH(CH_2)_fC{\equiv}CR^9$, and f is 1-20.

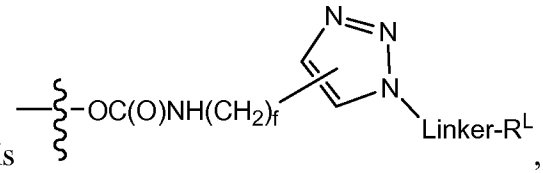
In one embodiment, R^1 is $-OC(O)NH(CH_2)_fC{\equiv}CR^9$, and f is 1-20.

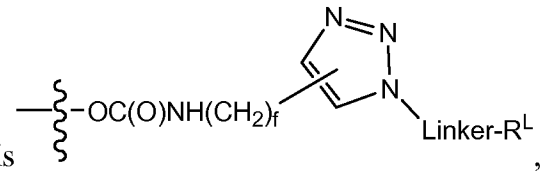
In one embodiment, B is a nucleobase substituted with $-OC(O)NH(CH_2)_fC{\equiv}CH$, and f is 1-20.

In one embodiment, R^5 is  , and f is 1-20.

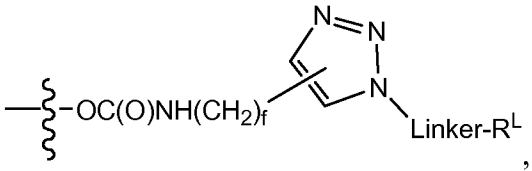
In one embodiment, R^4 is  , and f is 1-20.

In one embodiment, R^3 is  , and f is 1-20.

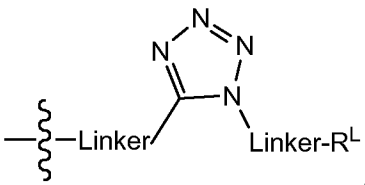
In one embodiment, R^2 is  , and f is 1-20.

In one embodiment, R^1 is  , and f is 1-20.

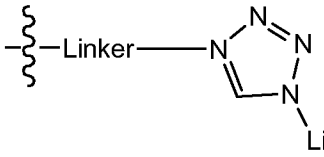
In one embodiment, B is a nucleobase substituted with

 , and f is 1-20.

In one embodiment, B is a nucleobase substituted with

 .

In one embodiment, B is a nucleobase substituted with



In one embodiment, R^5 is $-O-(CH_2)_fC\equiv CR^9$, and f is 1-20.

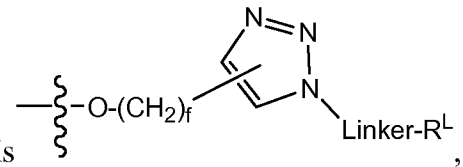
In one embodiment, R^4 is $-O-(CH_2)_fC\equiv CR^9$, and f is 1-20.

In one embodiment, R^3 is $-O-(CH_2)_fC\equiv CR^9$, and f is 1-20.

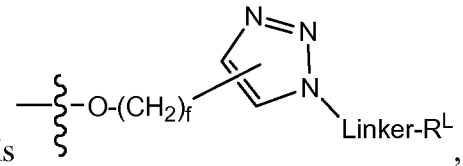
In one embodiment, R^2 is $-O-(CH_2)_fC\equiv CR^9$, and f is 1-20.

In one embodiment, R^1 is $-O-(CH_2)_fC\equiv CR^9$, and f is 1-20.

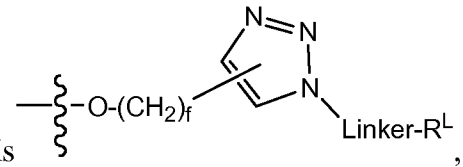
In one embodiment, B is a nucleobase substituted with $-O-(CH_2)_fC\equiv CH$, and f is 1-20

In one embodiment, R^5 is 

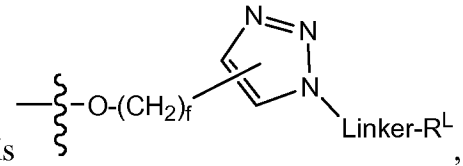
, and f is 1-20.

In one embodiment, R^4 is 

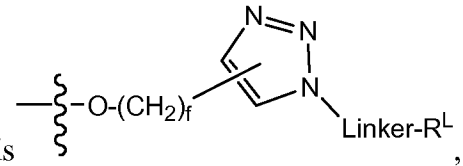
, and f is 1-20.

In one embodiment, R^3 is 

, and f is 1-20.

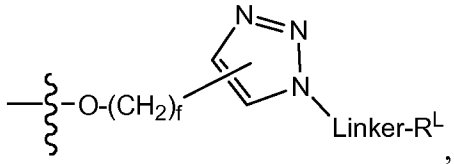
In one embodiment, R^2 is 

, and f is 1-20.

In one embodiment, R^1 is 

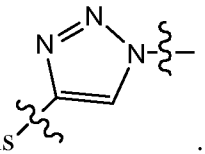
, and f is 1-20.

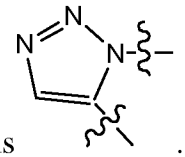
In one embodiment, B is nucleobase substituted with

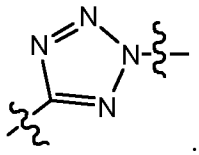


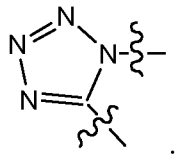
, and f is 1-20.

In one embodiment, f is 1, 2, 3, 4 or 5. In a preferred embodiment, f is 1.

In one embodiment Q is  .

In one embodiment, Q is  .

In one embodiment, Q is  .

In one embodiment, Q is  .

In one embodiment, when click-carrier compound of formula (I) is at the 5'-terminal end of an oligonucleotide, the oligonucleotides is linked at the R^5 position of the click-carrier compound.

In one embodiment, when click-carrier compound of formula (I) is at the 3'-terminal end of an oligonucleotide, the oligonucleotides is linked at the R^3 or R^2 position of the click-carrier compound.

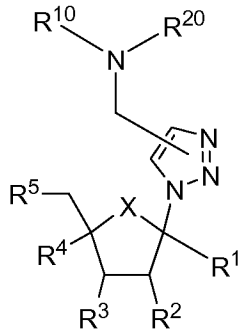
In one embodiment, when the click-carrier compound of formula (I) is not at a terminal position in an oligonucleotide, the R^5 position of the compound is linked to the

3'- or 2'-position of an oligonucleotide on one side and the R^2 or R^3 position of the compound is linked to the 5'-position of an oligonucleotide on the other side.

In one embodiment, the two different click-carrier compounds comprise complementary functional groups and are clicked together to each other. In one embodiment, complementarty functional groups are at R^5 position of one click-compound and R^2 or R^3 position of the second compound. In one embodiment, the complementarty functional groups are at R^5 position of one click-compound and R^5 position of the second compound. In one embodiment, complementarty functional groups are at R^2 or R^3 position of one click-compound and R^2 or R^3 position of the second compound.

In some embodiments, B can form part of the click-carrier that connects the linker to the carrier. For example, the –linker-Q-linker-R^L can be present at the C2, C6, C7 or C8 position of a purine nucleobase or at the C2, C5 or C6 position of a pyrimidine nucleobase. The linker can be directly attached to the nucleobase or indirectly through one or more intervening groups such as O, N, S, C(O), C(O)O C(O)NH. In certain embodiments, B, in the click-carrier described above, is uracilyl or a universal base, e.g., an aryl moiety, e.g., phenyl, optionally having additional substituents, e.g., one or more fluoro groups.

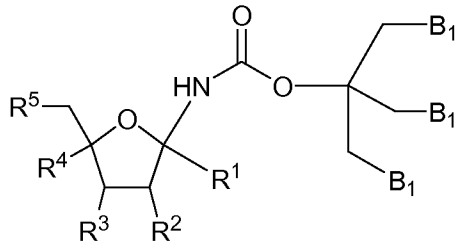
In one embodiment, the invention features a method for preparing a compound having the structure shown in formula (IV) by using a chelated copper catalyst of formula (A), (B), (C), or (D);



Formula (IV)

wherein R^{10} and R^{20} are independently for each occurrence hydrogen, optionally substituted aliphatic, optionally substituted aryl, or optionally substituted heteroaryl; R^1, R^2, R^3, R^4, R^5, and X are as defined in the first embodiment.

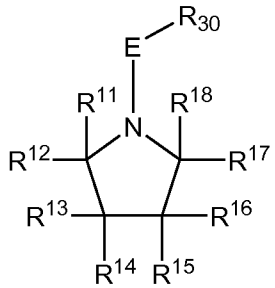
In one embodiment, the invention features a method for preparing a compound having the structure shown in formula (V) by using a chelated copper catalyst of formula (A), (B), (C), or (D);



Formula (V)

wherein B_1 is halogen, N_3, CN, optionally substituted triazole or optionally substituted tetrazole; R^1, R^2, R^3, R^4, and R^5 are as defined in the first embodiment.

In one embodiment, the invention features a method for preparing a compound having the structure shown in formula (VI) by using a chelated copper catalyst of formula (A), (B), (C), or (D);



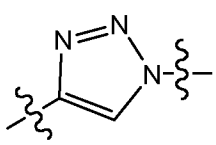
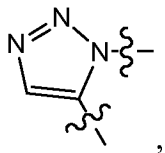
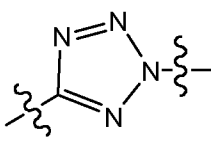
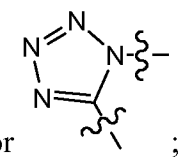
Formula (VI)

wherein E is absent or C(O), C(O)O, C(O)NH, C(S), C(S)NH, SO, SO_2, or SO_2NH;

R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, R^{16}, R^{17}, and R^{18} are each independently for each occurrence H, $-CH_2OR^a$, or OR^b,

R^a and R^b are each independently for each occurrence hydrogen, hydroxyl protecting group, optionally substituted alkyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted alkenyl, optionally substituted heteroaryl, polyethyleneglycol (PEG), a phosphate, a diphosphate, a triphosphate, a phosphonate, a phosphonothioate, a phosphonodithioate, a phosphorothioate, a phosphorothiolate, a phosphorodithioate, a phosphorothiolothionate, a phosphodiester, a phosphotriester, an activated phosphate group, an activated phosphite group, a phosphoramidite, a solid support, -P(Z^1)(Z^2)-O-nucleoside, -P(Z^1)(Z^2)-O-oligonucleotide, -P(Z^1)(Z^2)-formula (I), -P(Z^1)(O-linker-Q-linker-R^L)-O-nucleoside, -P(Z^1)(O-linker-N_3)-O-nucleoside, P(Z^1)(O-linker-CN)-O-nucleoside, P(Z^1)(O-linker-C≡R^8)-O-nucleoside, P(Z^1)(O-linker-cycloalkyne)-O-nucleoside, -P(Z^1)(O- linker-R^L)-O-oligonucleotide, -P(Z^1)(O-linker-Q-linker-R^L)-O-oligonucleotide, -P(Z^1)(O-linker-R^L)-O-oligonucleotide, P(Z^1)(O-linker-N_3)-O-oligonucleotide, -P(Z^1)(O-linker-CN)-O-oligonucleotide, P(Z^1)(O-linker-C≡R^8)-O-oligonucleotide, P(Z^1)(O-linker-cycloalkyne)-O-oligonucleotide, -P(Z^1)(-linker-Q-linker-R^L)-O-nucleoside, P(Z^1)(-linker- R^L)-O-nucleoside, -P(Z^1)(-linker-N_3)-O-nucleoside, P(Z^1)(-linker-CN)-O-nucleoside, P(Z^1)(-linker-C≡R^8)-O-nucleoside, P(Z^1)(-linker-cycloalkyne)-O-nucleoside, -P(Z^1)(-linker-Q-linker-R^L)-O-oligonucleotide, (Z^1)(-linker- R^L)-O-oligonucleotide, P(Z^1)(-linker-N_3)-O-oligonucleotide, -P(Z^1)(-linker-CN)-O-oligonucleotide, P(Z^1)(-linker-C≡R^8)-O-oligonucleotide or P(Z^1)(-linker-cycloalkyne)-O-oligonucleotide;

R^{30} is independently for each occurrence –linker-Q-linker-R^L, –linker-R^L or R^{31};

Q is absent or independently for each occurrence  ,  ,  , or  ;

R^L is hydrogen or a ligand;

R^8 is N or CR^9;

R^9 is H, optionally substituted alkyl or silyl;

R^{31} is $-C(O)CH(N(R^{32})_2)(CH_2)_hN(R^{32})_2$;

R^{32} is independently for each occurrence H, -linker-Q-linker-R^L, -linker- R^L or R^{31};

f and h are independently for each occurrence 1 -20; and

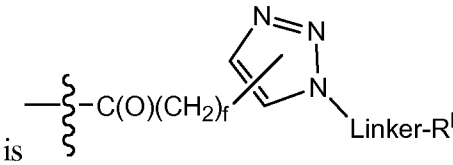
Z^1 and Z^2 are each independently for each occurrence O, S or optionally substituted alkyl.

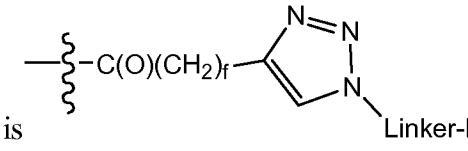
For the pyrroline-based click-carriers, R^{11} is $-CH_2OR^a$ and R^3 is OR^b; or R^{11} is $-CH_2OR^a$ and R^9 is OR^b; or R^{11} is $–CH_2OR^a$ and R^{17} is OR^b; or R^{13} is $–CH_2OR^a$ and R^{11} is OR^b; or R^{13} is $–CH_2OR^a$ and R^{15} is OR^b; or R^{13} is $–CH_2OR^a$ and R^{17} is OR^b. In certain embodiments, CH_2OR^a and OR^b may be geminally substituted. For the 4-hydroxyproline-based carriers, R^{11} is $-CH_2OR^a$ and R^{17} is OR^b. The pyrroline- and 4-hydroxyproline-based compounds 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_2OR^a and OR^b 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 compounds 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 compounds are expressly included (e.g., the centers bearing CH_2OR^a and OR^b 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*).

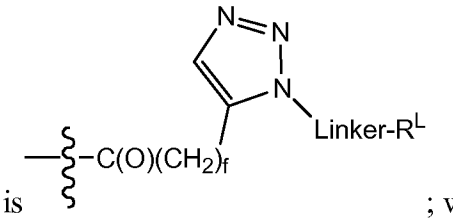
In one embodiment, R^{11} is CH_2OR^a and R^9 is OR^b.

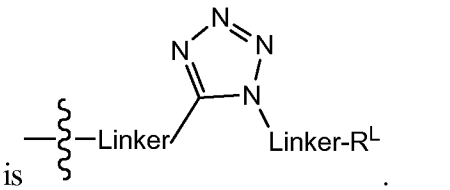
In one embodiment, R^b is a solid support.

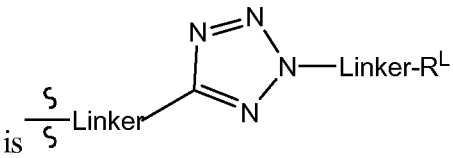
In one embodiment, R^{30} is $–C(O)(CH_2)_fNHC(O)(CH_2)_gC{\equiv}CR^9$; wherein f and g are independently 1 – 20.

In one embodiment, R^{30} is  ; wherein f is 1-20.

In one embodiment, R^{30} is  ; wherein f is 1-20.

In one embodiment, R^{30} is  ; wherein f is 1-20.

In one embodiment, R^{30} is  .

In one embodiment, R^{30} is  .

In one preferred embodiment, R^{30} is R^{31}.

In one preferred embodiment, R^{31} is $-C(O)CH(N(R^{32})_2)(CH_2)_4N(R^{32})_2$ and at least one R^{32} is $-C(O)(CH_2)_fC{\equiv}R^8$ or –linker-Q-linker-R^L and R^L is present.

In one preferred embodiment, R^{31} is $-C(O)CH(N(R^{32})_2)(CH_2)_4NH_2$ and at least one R^{32} is $-C(O)(CH_2)_fC{\equiv}R^8$ or –linker-Q-linker-R^L and R^L is present.

In one embodiment, R^{32} is $-C(O)(CH_2)_fC{\equiv}R^8$.

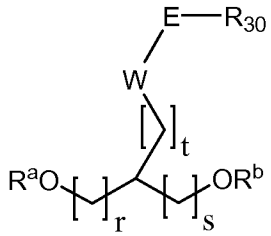
In one embodiment, R^{32} is $-C(O)(CH_2)_3C{\equiv}H$.

In one embodiment, R^{31} is $-C(O)CH(NH_2)(CH_2)_4NH_2$.

In one embodiment features acyclic sugar replacement-based compounds, e.g., sugar replacement based click-carrier compounds, are also referred to herein as ribose

replacement compound subunit (RRMS) compound compounds. Preferred acyclic carriers can have the structure shown in formula (III) or formula (IV) below.

In one aspect, the invention features a method for preparing a compound having the structure shown in formula (VII) by using a chelated copper catalyst of formula (A), (B), (C), or (D);



Formula (VII)

wherein:

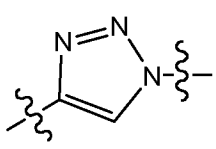
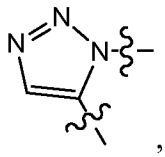
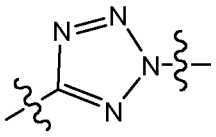
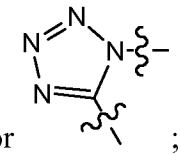
W is absent, O, S and $N(R^N)$, where R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group;

E is absent or C(O), C(O)O, C(O)NH, C(S), C(S)NH, SO, SO_2, or SO_2NH;

R^a and R^b are each independently for each occurrence hydrogen, hydroxyl protecting group, optionally substituted alkyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted alkenyl, optionally substituted heteroaryl, polyethyleneglycol (PEG), a phosphate, a diphosphate, a triphosphate, a phosphonate, a phosphonothioate, a phosphonodithioate, a phosphorothioate, a phosphorothiolate, a phosphorodithioate, a phosphorothiolothionate, a phosphodiester, a phosphotriester, an activated phosphate group, an activated phosphite group, a phosphoramidite, a solid support, -$P(Z^1)(Z^2)$-O-nucleoside, -$P(Z^1)(Z^2)$-O-oligonucleotide, -$P(Z^1)(Z^2)$-formula (I), -$P(Z^1)$(O-linker-Q-linker-R^L)-O-nucleoside, -$P(Z^1)$(O-linker-N_3)-O-nucleoside, $P(Z^1)$(O-linker-CN)-O-nucleoside, $P(Z^1)$(O-linker-$C{\equiv}R^8$)-O-nucleoside, $P(Z^1)$(O-linker-cycloalkyne)-O-nucleoside, -$P(Z^1)$(O- linker-R^L)-O-oligonucleotide, -$P(Z^1)$(O-linker-Q-linker-R^L)-O-oligonucleotide, -$P(Z^1)$(O-linker-R^L)-O-

oligonucleotide, $P(Z^1)$(O-linker-N_3)-O-oligonucleotide, -$P(Z^1)$(O-linker-CN)-O-oligonucleotide, $P(Z^1)$(O-linker-C≡R^8)-O-oligonucleotide, $P(Z^1)$(O-linker-cycloalkyne)-O-oligonucleotide, -$P(Z^1)$(-linker-Q-linker-R^L)-O-nucleoside, $P(Z^1)$(-linker- R^L)-O-nucleoside, -$P(Z^1)$(-linker-N_3)-O-nucleoside, $P(Z^1)$(-linker-CN)-O-nucleoside, $P(Z^1)$(-linker-C≡R^8)-O-nucleoside, $P(Z^1)$(-linker-cycloalkyne)-O-nucleoside, -$P(Z^1)$(-linker-Q-linker-R^L)-O-oligonucleotide, (Z^1)(-linker- R^L)-O-oligonucleotide, $P(Z^1)$(-linker-N_3)-O-oligonucleotide, -$P(Z^1)$(-linker-CN)-O-oligonucleotide, $P(Z^1)$(-linker-C≡R^8)-O-oligonucleotide or $P(Z^1)$(-linker-cycloalkyne)-O-oligonucleotide;

R^{30} is independently for each occurrence –linker-Q-linker-R^L, –linker-R^L or R^{31};

Q is absent or independently for each occurrence  ,  ,  , or  ;

R^L is hydrogen or a ligand;

R^8 is N or CR^9

R^9 is H, optionally substituted alkyl or silyl;

R^{31} is -C(O)CH(N(R^{32})$_2$)(CH_2)$_h$N(R^{32})$_2$;

R^{32} is independently for each occurrence H, -linker-Q-linker-R^L or R^{31};

f and h are independently for each occurrence 1 -20;

Z^1 and Z^2 are each independently for each occurrence O, S or optionally substituted alkyl; and

r, s and t are each independently for each occurrence 0, 1, 2 or 3.

When r and s are different, then the tertiary carbon can be either the R or S configuration. In preferred embodiments, x and y are one and z is zero (e.g. carrier is

based on serinol). The acyclic carriers can optionally be substituted, e.g. with hydroxy, alkoxy, perhaloalky.

Other carrier compounds amenable to the invention are described in copending applications USSN: 10/916,185, filed August 10, 2004; USSN: 10/946,873, filed September 21, 2004; USSN: 10/985,426, filed November 9, 2004; USSN: 10/833,934, filed August 3, 2007; USSN: 11/115,989 filed April 27, 2005 and USSN: 11/119,533, filed April 29, 2005, which are incorporated by reference in their entireties for all purposes.

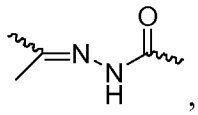
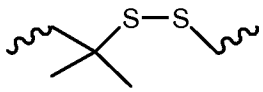
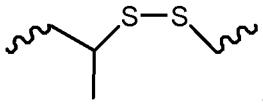
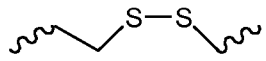
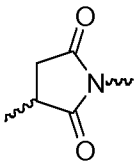
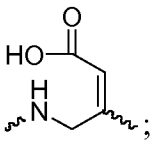
LINKERS

The term "linker" means an organic moiety that connects two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR^1, C(O), C(O)NH, SO, SO_2, SO_2NH or a chain of atoms, such as substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylhererocyclylalkynyl, alkenylheterocyclylalkyl, alkenylheterocyclylalkenyl, alkenylheterocyclylalkynyl, alkynylheterocyclylalkyl, alkynylheterocyclylalkenyl, alkynylheterocyclylalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylhereroaryl, which one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, $N(R^1)_2$, C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R^1 is hydrogen, acyl, aliphatic or substituted aliphatic. It is understood that a linker can cleavable or non-cleavable.

In one embodiment, the linker is represented by structure

$$-[P\text{-}Q_1\text{-}R]_q\text{-}T\text{-},$$

wherein:

P, R and T are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH_2, CH_2NH, CH_2O; $NHCH(R^a)C(O)$, -C(O)-$CH(R^a)$-NH-, -C(O)-(optionally substituted alkyl)-NH-, CH=N-O ,  ,  ,  ,  ,  or  ;

Q_1 is independently for each occurrence absent, $-(CH_2)_n-$, $-C(R^{100})(R^{200})(CH_2)_n-$, $-(CH_2)_nC(R^{100})(R^{200})-$, $-(CH_2CH_2O)_mCH_2CH_2-$, or $-(CH_2CH_2O)_mCH_2CH_2NH-$;

R^a is H or an amino acid side chain;

R^{100} and R^{200} are each independently for each occurrence H, CH_3, OH, SH or $N(R^X)_2$;

R^X is independently for each occurrence H, methyl, ethyl, propyl, isopropyl, butyl or benzyl;

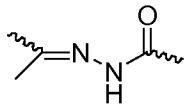
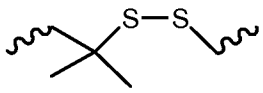
q is independently for each occurrence 0-20;

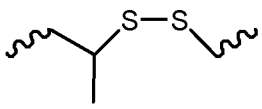
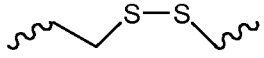
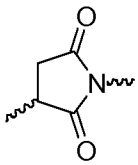
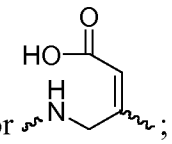
n is independently for each occurrence 1-20; and

m is independently for each occurrence 0-50.

In one embodiment, the linker has the structure $-[(P\text{-}Q_1\text{-}R)_q\text{-}X\text{-}(P'\text{-}Q_1'\text{-}R')_{q'}]_{q''}\text{-}T$, wherein:

P, R, T, P', R' and T' are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH_2, CH_2NH, CH_2O; $NHCH(R^a)C(O)$, -C(O)-$CH(R^a)$-NH-,

-C(O)-(optionally substituted alkyl)-NH-, CH=N-O ,  ,  ,

S-S, acetal, ketal,  ,  ,  or  ;

Q_1 and Q_1' are each independently for each occurrence absent, $-(CH_2)_n-$, $-C(R^{100})(R^{200})(CH_2)_n-$, $-(CH_2)_nC(R^{100})(R^{200})-$, $-(CH_2CH_2O)_mCH_2CH_2-$, or $-(CH_2CH_2O)_mCH_2CH_2NH-$;

X is a cleavable linker;

R^a is H or an amino acid side chain;

R^{100} and R^{200} are each independently for each occurrence H, CH_3, OH, SH or $N(R^X)_2$;

R^X is independently for each occurrence H, methyl, ethyl, propyl, isopropyl, butyl or benzyl;

q, q' and q' are each independently for each occurrence 0-20;

n is independently for each occurrence 1-20; and

m is independently for each occurrence 0-50.

In one embodiment, the linker comprises at least one cleavable linker.

Cleavable Linkers

A cleavable linker is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linker is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

Cleavable linkers 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 linker 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 linker by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

A cleavable linker, 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 sapcers will have a linker that is cleaved at a preferred pH, thereby releasing the iRNA agent from the carrier oligomer inside the cell, or into the desired compartment of the cell.

A spacer can include a linker that is cleavable by a particular enzyme. The type of linker incorporated into a spacer 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 linked to the carrier oligomer through a spacer 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 sapcer releases the iRNA agent from the carrier oligomer, thereby potentially enhancing silencing activity of the iRNA agent. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

Spacers that contain peptide bonds can be used when the iRNA agents are targeting 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 linked to a carrier oligomer through spacer that comprises a peptide bond.

In general, the suitability of a candidate cleavable linker can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linker. It will also be desirable to also test the candidate cleavable linker 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 linkers

One class of cleavable linkers are redox cleavable linkers that are cleaved upon reduction or oxidation. An example of reductively cleavable linker is a disulphide linker (-S-S-). To determine if a candidate cleavable linker is a suitable "reductively cleavable linker," 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 linkers

Phosphate-based linkers 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 linkers 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 linkers

Acid cleavable linkers are linkers that are cleaved under acidic conditions. In preferred embodiments acid cleavable linkers 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 linkers. Examples of acid cleavable linkers include but are not limited to hydrazones, 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 linkers

Ester-based linkers are cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linkers include but are not limited to esters of

alkylene, alkenylene and alkynylene groups. Ester cleavable linkers 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

Peptide-based linkers are cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linkers 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 linkers 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.

"Click" reaction

The synthesis methods of the present invention utilize click chemistry to conjugate the ligand to the click-carrier compound. Click chemistry techniques are described, for example, in the following references, which are incorporated herein by reference in their entirety:

Kolb, H. C.; Finn, M. G. and Sharpless, K. B. *Angew. Chem., Int. Ed.* (2001) 40: 2004-2021.

Kolb, H. C. and Shrapless, K. B. *Drug Disc. Today* (2003) 8: 112-1137.

Rostovtsev, V. V.; Green L. G.; Fokin, V. V. and Shrapless, K.B. *Angew. Chem., Int. Ed.* (2002) 41: 2596-2599.

Tornøe, C. W.; Christensen, C. and Meldal, M. *J. Org. Chem.* (2002) 67: 3057-3064.

Wang, Q. *et al., J. Am. Chem. Soc.* (2003) 125: 3192-3193..
Lee, L.V.*et al., J. Am. Chem. Soc.* (2003) 125: 9588-9589.
Lewis, W.G. *et al., Angew. Chem., Int. Ed.* (2002) 41: 1053-1057.
Manetsch, R. *et al., J. Am. Chem. Soc.* (2004) 126: 12809-12818.
Mocharla, V.P. *et al., Angew. Chem., Int. Ed.* (2005) 44: 116-120.

Although other click chemistry functional groups can be utilized, such as those described in the above references, the use of cycloaddition reactions is preferred, particularly the reaction of azides with alkynyl groups. In the presence of Cu(I) salts, terminal alkynes and azides undergo 1,3-dipolar cycloaddition forming 1,4-disubsstituted 1,2,3-triazoles. In the presence of Ru(II) salts (e.g. Cu*RuCl$(PPh_3)_2$), terminal alkynes and azides under go 1,3-dipolar cycloaddition forming 1,5-disubstituted 1,2,3-triazoles (Folkin, V.V. *et al., Org. Lett.* (2005) 127: 15998-15999). Alternatively, a 1,5-disubstituted 1,2,3-triazole can be formed using azide and alkynyl reagents (Kraniski, A.; Fokin, V.V. and Sharpless, K.B. *Org. Lett.* (2004) 6: 1237-1240. Hetero-Diels-Alder reactions or 1,3-dipolar cycloaddition reaction could also be used (see for example Padwa, A. 1,3-*Dipolar Cycloaddition Chemistry: Volume 1,* John Wiley, New York, (1984) 1-176; Jørgensen, K. A. *Angew. Chem., Int. Ed.* (2000) 39: 3558-3588 and Tietze, L. F. and Kettschau, G. *Top. Curr. Chem.* (1997) 189: 1-120)

The choice of azides and alkynes as coupling partners is particularly advantageous as they are essentially non-reactive towards each other (in the absence of copper) and are extremely tolerant of other functional groups and reaction conditions. This chemical compatibility helps ensure that many different types of azides and alkynes may be coupled with each other with a minimal amount of side reactions.

The required copper(I) species are added directly as cuprous salts, for example CuI, CuOTf.C_6H_6 or $[Cu(CH_3CN)_4][PF_6]$, usually with stabilizing ligands (see for example Tornøe, C. W.; Christensen, C. and Meldal, M. *J. Org. Chem.* (2002) 67: 3057-3064; Chan, T. R. *et al., Org. Lett.* (2004) 6: 2853-2855; Lewis, W.G. *et al., J. Am. Chem. Soc.* (2004) 126: 9152-9153; Mantovani, G. *et al., Chem. Comm.* (2005) 2089-2091; Diez-Gonzalez, S. *et al., Chem. Eur. J.* (2006) 12: 7558-7564 and Candelon, N. *et*

al., Chem. Comm. (2008) 741-743), or more often generated from copper (II) salts with reducing agents (Rostovtsev, V. V. *et al., Angew. Chem.* (2002) 114: 2708—2711 and *Angew. Chem., Int. Ed.* (2002) 41: 2596-2599). Metallic copper (for example see Himo, F. *et al., J. Am. Chem. Soc.* (2005) 127: 210-216) or clusters (for example see Pachon, L. D. *et al., Adv. Synth. Catal.* (2005) 347: 811-815 and Molteni, G. *et al., New J. Chem* (2006) 30: 1137-1139) can also be employed. Chassaing *et al.,* recently reported copper(I) zeolites as catalysts for the azide-alkyne cycloaddition (*Chem. Eur. J.* (2008) 14: 6713-6721). As copper(I) salts are prone to redox process, nitrogen- or phosphorous-based ligands must be added to protect and stabilize the active copper catalyst during the cycloaddition reaction.

The reaction is extremely straightforward. The azide and alkyne are usually mixed together in water and a co-solvent such as *tert*-butanol, THF, DMSO, toluene or DMF. The water/co-solvent are usually in a 1:1 to 1:9 ratio. The reactions are usually run overnight although mild heating shortens reaction times (Sharpless, W. D.; Wu, P.; Hansen, T. V.; and Li, J.G. *J. Chem. Ed.* (2005) 82: 1833). Aqueous systems can also use copper(I) species directly such that a reducing agent is not needed. The reactions conditions then usually require acetonitrile as a co-solvent (although not essential (Chan, T. R.; Hilgraf, R.; Shrapless, K. B. and Fokin, V.V. *Org. Lett.* (2004) 6: 2853)) and a nitrogen base, such as triethylamine, 2,6-lutidine, pyridine and diisopropylamine. In this case copper(I) species is supplied as CuI, $CuOTf.C_6H_6$ or $[Cu(CH_3CN)_4][PF_6]$ (Rostoctsev, V. V.; Green L. G.; Fokin, V. V. and Shrapless, K.B. *Angew. Chem., Int. Ed.* (2002) 41: 2596-2599).

Although the water-based methods are attractive for many applications, solvent based azide-alkyne cycloaddition methods have found utility in situations when solubility and/or other problems arise, for example see:

Malkoch, M. *et al., Macromolecules* (2005) 38: 3663.

Gujadhur, R.; Venkataraman, D. and J. T. Kintigh. J.T. *Tet. Lett.* (2001) 42: 4791.

Laurent, B.A. and Grayson, S.M. *J. Am. Chem. Soc.* (2006) 128: 4238.

Opsteen, J. A.; van Hest, J. C. M. *Chem. Commun.* (2005) 57.

Tsarevsky, N. V.; Sumerlin, B. S. and Matyjaszewski, K. *Macromolecules* (2005) 38: 3558.

Johnson, J. A. *et al., J. Am. Chem. Soc.* (2006) 128: 6564.

Sumerlin, B. S. *et al.*, *Macromolecules* (2005) 38: 7540.

Gao, H. F. and Matyjaszewski, K. *Macromolecules* (2006) 39: 4960.

Gao, H. *et al, Macromolecules* (2005) 38: 8979.

Vogt, A. P. and Sumerlin, B.S. *Macromolecules* (2006) 39: 5286.

Lutz, J. F.; Borner, H. G. and Weichenhan, K. *Macromol. Rapid Comm.* (2005) 26: 514.

Mantovani, G.; Ladmiral, V.; Tao, L. and Haddleton, D. M. *Chem. Comm.* (2005) 2089.

The click reaction may be performed thermally. In one aspect, the click reaction is performed at slightly elevated temperatures between 25°C and 100°C. In one aspect, the reaction may be performed between 25°C and 75°C, or between 25°C and 65°C, or between 25°C and 50°C. In one embodiment, the reaction is performed at room temperature. In another aspect, the click reaction may also be performed using a microwave oven. The microwave assisted click reaction may be carried out in the presence or absence of copper.

In one aspect, the invention provides a method for coupling a click-carrier compound to a ligand through a click reaction. In a preferred embodiment, the click reaction is a cycloaddition reaction of azide with alkynyl group and catalyzed by copper. In one embodiment the equal molar amount of alkyne and azide are mixed together in DCM/MeOH (10:1 to 1:1 ratio v/v) and 0.05-0.5 mol% each of $[Cu(CH_3CN)_4][PF_6]$ and copper are added the reaction. In one embodiment DCM/MeOH ratio is 5:1 to 1:1. In a preferred embodiment, DCM/MeOH ratio is 4:1. In one embodiment, equal molar amounts of $[Cu(CH_3CN)_4][PF_6]$ and copper are added. In a preferred embodiment, 0.05-0.25mol% each of $[Cu(CH_3CN)_4][PF_6]$ and copper are added to the reaction. In a more preferred embodiment, 0.05 mol%, 0.1 mol%, 0.15 mol%, 0.2 mol% or 0.25 mol% each of $[Cu(CH_3CN)_4][PF_6]$ and copper are added to the reaction.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl)phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published Dec. 9, 1993 or in WO 94/26764 and U.S. Pat. No. 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the oligomeric compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. For oligonucleotides, preferred examples of pharmaceutically acceptable salts and their uses are further described in U.S. Pat. No. 6,287,860, which is incorporated herein in its entirety.

Ligands

A wide variety of entities can be coupled to the oligonucleotide, e.g. the iRNA agent, using the "click" reaction. Preferred entities can be coupled to the oligonucleotide at various places, for example, 3'-end, 5'-end, and/or at internal positions.

In preferred embodiments, the ligand is attached to the iRNA agent *via* an intervening linker. The ligand may be present on a compound when said compound is incorporated into the growing strand. In some embodiments, the ligand may be incorporated via coupling to a "precursor" compound after said "precursor" compound has been incorporated into the growing strand. For example, a compound having, e.g., an azide terminated linker (i.e., having no associated ligand), e.g., -linker-N_3 may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor compound into the strand, a ligand having an alkyne, e.g. terminal acetylene, e.g. ligand-C≡CH, can subsequently be attached to the precursor compound by the "click" reaction. Alternatively, the compound linker comprises an alkyne, e.g. terminal acetylene; and the ligand comprises an azide functionality for the "click" reaction to take place. The azide or alkyne functionalities can be incorporated

into the ligand by methods known in the art. For example, moieties carrying azide or alkyne functionalities can be linked to the ligand or a functional group on the ligand can be transformed into an azide or alkyne. In one embodiment, the conjugation of the ligand to the precursor compound takes place while the oligonucleotide is still attached to the solid support. In one embodiment, the precursor carrying oligonucleotide is first deprotected but not purified before the ligand conjugation takes place. In one embodiment, the precursor compound carrying oligonucleotide is first deprotected and purified before the ligand conjugation takes place. In certain embodiments, the "click" reaction is carried out under microwave.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand. Preferred ligands will not take part in duplex pairing in a duplexed nucleic acid.

Preferred ligands can have endosomolytic properties. The endosomolytic ligands promote the lysis of the endosome and/or transport of the composition of the invention, or its components, from the endosome to the cytoplasm of the cell. The endosomolytic ligand may be a polyanionic peptide or peptidomimetic which shows pH-dependent membrane activity and fusogenicity. In certain embodiments, the endosomolytic ligand assumes its active conformation at endosomal pH. The "active" conformation is that conformation in which the endosomolytic ligand promotes lysis of the endosome and/or transport of the composition of the invention, or its components, from the endosome to the cytoplasm of the cell. Exemplary endosomolytic ligands include the GALA peptide (Subbarao et al., Biochemistry, 1987, 26: 2964-2972), the EALA peptide (Vogel et al., J. Am. Chem. Soc., 1996, 118: 1581-1586), and their derivatives (Turk et al., Biochem. Biophys. Acta, 2002, 1559: 56-68). In certain embodiments, the endosomolytic component may contain a chemical group (e.g., an amino acid) which will undergo a change in charge or protonation in response to a change in pH. The endosomolytic

component may be linear or branched. Exemplary primary sequences of peptide based endosomolytic ligands are shown in table 1.

Table 1: List of peptides with endosomolytic activity.

Name	Sequence (N to C)	Ref.
GALA	AALEALAEALEALAEALEALAEAAAAGGC	1
EALA	AALAEALAEALAEALAEALAEALAAAAGGC	2
	ALEALAEALEALAEA	3
INF-7	GLFEAIEGFIENGWEGMIWDYG	4
Inf HA-2	GLFGAIAGFIENGWEGMIDGWYG	5
diINF-7	GLF EAI EGFI ENGW EGMI DGWYGC GLF EAI EGFI ENGW EGMI DGWYGC	5
diINF3	GLF EAI EGFI ENGW EGMI DGGC GLF EAI EGFI ENGW EGMI DGGC	6
GLF	GLFGALAEALAEALAEHLAEALAEALEALAAGG SC	6
GALA-INF3	GLFEAIEGFIENGWEGLAEALAEALEALAAGGSC	6
INF-5	GLF EAI EGFI ENGW EGnI DG K GLF EAI EGFI ENGW EGnI DG	4

n, norleucine

References

1. Subbarao *et al.* (1987) *Biochemistry* 26: 2964-2972.
2. Vogel, *et al.* (1996) *J. Am. Chem. Soc.* 118: 1581-1586
3. Turk, *et al.* (2002) *Biochim. Biophys. Acta* 1559: 56-68.
4. Plank, *et al.* (1994) *J. Biol. Chem.* 269:12918–12924.
5. Mastrobattista, *et al.* (2002) *J. Biol. Chem.* 277:27135-43.
6. Oberhauser, *et al.* (1995) *Deliv. Strategies Antisense Oligonucleotide Ther.* 247-66.

Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of compounds described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; cross-linking

agents; and nuclease-resistance conferring moieties. General examples include lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, and peptide mimics.

Ligands can include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); an carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a 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), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer. Table 2 shows some examples of targeting ligands and their associated receptors.

Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic molecules, e.g, 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)and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Table 2: Liver targeting Ligands and their associated receptors

Liver Cells	Ligand	Receptor
1) Parenchymal Cell (PC) (Hepatocytes)	Galactose	ASGP-R (Asiologlycoprotein receptor)
	Gal NAc (n-acetyl-galactosamine)	ASPG-R (GalNAc Receptor)
	Lactose	
	Asialofetuin	ASPG-r
2) Sinusoidal Endothelial Cell (SEC)	Hyaluronan	Hyaluronan receptor
	Procollagen	Procollagen receptor
	Negatively charged molecules	Scavenger receptors
	Mannose	Mannose receptors
	N-acetyl Glucosamine	Scavenger receptors
	Immunoglobulins	Fc Receptor
	LPS	CD14 Receptor
	Insulin	Receptor mediated transcytosis
	Transferrin	Receptor mediated transcytosis
	Albumins	Non-specific
	Sugar-Albumin conjugates	
	Mannose-6-phosphate	Mannose-6-phosphate receptor
3) Kupffer Cell (KC)	Mannose	Mannose receptors
	Fucose	Fucose receptors
	Albumins	Non-specific
	Mannose-albumin conjugates	

Ligands can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose,

multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, e.g, a drug, which can increase the uptake of the iRNA agent 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 ligand can increase the uptake of the iRNA agent into the cell by activating an inflammatory response, for example. Exemplary ligands that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HAS, low density lipoprotein (LDL) and high-density lipoprotein (HDL).

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

The ligand can be a peptide or peptidomimetic. 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 attachment of peptide and peptidomimetics to iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety 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 (see Table 3, for example).

Table 3. Exemplary Cell Permeation Peptides

Cell Permeation Peptide	Amino acid Sequence	Reference
Penetratin	RQIKIWFQNRRMKWKK	Derossi *et al.*, J. Biol. Chem. 269:10444, 1994

Tat fragment (48-60)	GRKKRRQRRRPPQC	Vives *et al.*, J. Biol. Chem., 272:16010, 1997
Signal Sequence-based peptide	GALFLGWLGAAGSTMGAWSQPKKKRKV	Chaloin *et al.*, Biochem. Biophys. Res. Commun., 243:601, 1998
PVEC	LLIILRRRIRKQAHAHSK	Elmquist *et al.*, Exp. Cell Res., 269:237, 2001
Transportan	GWTLNSAGYLLKINLKALAALAKKIL	Pooga *et al.*, FASEB J., 12:67, 1998
Amphiphilic model peptide	KLALKLALKALKAALKLA	Oehlke *et al.*, Mol. Ther., 2:339, 2000
Arg_9	RRRRRRRRR	Mitchell *et al.*, J. Pept. Res., 56:318, 2000
Bacterial cell wall permeating	KFFKFFKFFK	
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	
α-defensin	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	
b-defensin	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK)	
Bactenecin	RKCRIVVIRVCR	
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFPGKR-NH2	
Indolicidin	ILPWKWPWWPWRR-NH2	

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a

hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP. An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ) and the Drosophila Antennapedia protein (RQIKIWFQNRRMKWKK) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, Nature, 354:82-84, 1991). Preferably the peptide or peptidomimetic tethered to an iRNA agent via an incorporated compound unit is a cell targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

An RGD peptide moiety can be used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, Cancer Res., 62:5139-43, 2002). An RGD peptide can facilitate targeting of an iRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki *et al.*, Cancer Gene Therapy 8:783-787, 2001). Preferably, the RGD peptide will facilitate targeting of an iRNA agent to the kidney. The RGD peptide can be linear or cyclic, and can be modified, *e.g.,* glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver an iRNA agent to a tumor cell expressing $\alpha_V\beta_3$ (Haubner *et al.*, Jour. Nucl. Med., 42:326-336, 2001).

Peptides that target markers enriched in proliferating cells can be used. *E.g.*, RGD containing peptides and peptidomimetics can target cancer cells, in particular cells that exhibit an $I_v\vartheta_3$ integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD

mimics. In addition to RGD, one can use other moieties that target the I_v-ϑ_3 integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogeneis. Preferred conjugates of this type lignads that targets PECAM-1, VEGF, or other cancer gene, e.g., a cancer gene described herein.

Table 4. Azide modified peptides.

NB12675	N3-(CH2)5-CO-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln–NH2
NB12707	N3-(CH2)15-CO-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Pro-Gln–NH2
NB12676	cyclo-[Phe-Arg-Gly-Asp-Lys(N3-(CH2)5-COO H)]
NB12708	cyclo-[Phe-Arg-Gly-Asp-Lys(N3-(CH2)15-COOH)]
NB12709	N3-(CH2)5-CO-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg–NH2
NB12710	N3-(CH2)15-CO-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg–NH2

A "cell permeation peptide" is capable of permeating a cell, *e.g.,* a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*, α -defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, Nucl. Acids Res. 31:2717-2724, 2003).

In one embodiment, a targeting peptide tethered to an iRNA agent and/or the carrier oligomer can be an amphipathic α-helical peptide. Exemplary amphipathic α-helical 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. A number of factors will preferably be considered to maintain the integrity of helix stability.

For example, a maximum number of helix stabilization residues will be utilized (*e.g.*, leu, ala, or lys), and a minimum number helix destabilization residues will be utilized (*e.g.,* proline, or cyclic compoundic units. The capping residue will be considered (for example Gly is an exemplary N-capping residue and/or C-terminal amidation can be used to provide an extra H-bond to stabilize the helix. Formation of salt bridges between residues with opposite charges, separated by i ± 3, or i ± 4 positions can provide stability. For example, cationic residues such as lysine, arginine, homo-arginine, ornithine or histidine can form salt bridges with the anionic residues glutamate or aspartate.

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.

The targeting ligand can be any ligand that is capable of targeting a specific receptor. Examples are: folate, GalNAc, $GalNAc_3$, galactose, mannose, mannose-6P, clusters of sugars such as GalNAc cluster, mannose cluster, galactose cluster, or an apatamer. A cluster is a combination of two or more sugar units. The targeting ligands also include integrin receptor ligands, Chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL and HDL ligands. The ligands can also be based on nucleic acid, e.g., an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

Endosomal release agents include imidazoles, poly or oligoimidazoles, PEIs, peptides, fusogenic peptides, polycaboxylates, polyacations, masked oligo or poly cations or anions, acetals, polyacetals, ketals/polyketyals, orthoesters, polymers with masked or unmasked cationic or anionic charges, dendrimers with masked or unmasked cationic or anionic charges.

PK modulator stands for pharmacokinetic modulator. PK modulator include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins etc. Examplary PK modulator include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin etc.

Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, e.g. oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the backbaone are also amenable to the present invention as ligands (e.g. as PK modulating ligands).

In addition, aptamers that bind serum components (e.g. serum proteins) are also amenable to the present invention as PK modulating ligands.

Other ligands amenable to the invention are described in copending applications USSN: 10/916,185, filed August 10, 2004; USSN: 10/946,873, filed September 21, 2004; USSN: 10/833,934, filed August 3, 2007; USSN: 11/115,989 filed April 27, 2005 and USSN: 11/944,227 filed November 21, 2007, which are incorporated by reference in their entireties for all purposes.

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.

The compound comprising the ligand, e.g. the click-carrier compound, can be present in any position of an oligonucleotide, e.g. an iRNA agent. In some embodiments, click-carrier compound can be present at the terminus such as a 5' or 3' terminal of the iRNA agent. Click-carrier compounds can also present at an internal postion of the iRNA agent. For double- stranded iRNA agents, click-carrier compounds can be incorporated into one or both strands. In some embodiments, the sense strand of the double-stranded iRNA agent comprises the click-carrier compound. In other embodiments, the antisense strand of the double-stranded iRNA agent comprises the click-carrier compound.

In some embodiments, lignads can be conjugated to nucleobases, sugar moieties, or internucleosidic linkages of nucleic acid molecules. 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. Conjugation to sugar moieties of nucleosides can occur at any carbon atom. Example 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, phosphorodithiotate, 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.

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

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 oligomeric compounds with and without linkers 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.

Representative United States patents that teach the preparation of oligonucleotide conjugates 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; each of which is herein incorporated by reference.

Oligonucleotides

In the context of this invention, the term "oligonucleotide" refers to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term "oligonucleotide" also includes polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

The nucleic acids used herein can be single-stranded or double-stranded. A single stranded oligonucleotide may have double stranded regions and a double stranded oligonucleotide may have regions of single-stranded regions. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA include siRNA and other RNA interference reagents. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, microRNAs, aptamers, antagomirs, triplex-forming oligonucleotides and single-stranded RNAi agents.

Oligonucleotides of the present invention may be of various lengths. In particular embodiments, oligonucleotides may range from about 10 to 100 nucleotides in length. In various related embodiments, oligonucleotides, single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 50 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, from about 20 to about 30 nucleotides in length.

The oligonucleotides of the invention may comprise any oligonucleotide modification described herein and below. In certain instances, it may be desirable to modify one or both strands of a dsRNA. In some cases, the two strands will include different modifications. Multiple different modifications can be included on each of the strands. The modifications on a given strand may differ from each other, and may also differ from the various modifications on other strands. For example, one strand may have a modification, e.g., a modification described herein, and a different strand may have a different modification, e.g., a different modification described herein. In other cases, one strand may have two or more different modifications, and the another strand may include a modification that differs from the at least two modifications on the other strand.

In one embodiment, oligonucleotides of the invention comprises 5' phosphorothioate or 5'-phosphorodithioate, nucleotides 1 and 2 having cationic modifications via C-5 position of pyrimidines, 2-Position of Purines, N2-G, G-clamp, 8-position of purines, 6-position of purines, internal nucleotides having a 2'-F sugar with base modifications (Pseudouridine, G-clamp, phenoxazine, pyridopyrimidines, gem2'-Me-up/2'-F-down), 3'-end with two purines with novel 2'-substituted MOE analogs, 5'-end nucleotides with novel 2'-substituted MOE analogs, 5'-end having a 3'-F and a 2'-5'-linkage, 4'-substituted nucleoside at the nucleotide 1 at 5'-end and the substituent is cationic, alkyl, alkoxyalkyl, thioether and the like , 4'-substitution at the 3'-end of the strand, and combinations thereof.

Double-stranded oligonucleotides

In one embodiment, the invention provides double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of the target gene (alone or in combination with a second dsRNA for inhibiting the expression of a second target gene) in a cell or mammal, wherein the dsRNA comprises an antisense strand comprising a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of the target gene, and wherein the region of complementarity is less than 30 nucleotides in length, generally 19-24 nucleotides in length, and wherein said dsRNA, upon contact with a cell expressing said target gene, inhibits the expression of

said target gene. The dsRNA comprises two RNA 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 one embodiment, longer dsRNAs of between 25 and 30 base pairs in length are preferred. In one embodiment, shorter dsRNAs of between 10 and 15 base pairs in length are preferred. In another embodiment, the dsRNA is at least 21 nucleotides long and includes a sense RNA strand and an antisense RNA strand, wherein the antisense RNA strand is 25 or fewer nucleotides in length, and the duplex region of the dsRNA is 18-25 nucleotides in length, e.g., 19-24 nucleotides in length.

Similarly, the region of complementarity to the target sequence 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 nucleotides in length. The dsRNA of the invention may further comprise one or more single-stranded nucleotide overhang(s).

In a preferred embodiment, the target gene is a human target gene. In one embodiment, the target gene is selected from the group consisting of Factor VII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, 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, 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, RAS gene, caveolin I gene, MIB I gene, MTAI gene, M68 gene, mutations in tumor suppressor genes, p53 tumor suppressor gene, and combinations thereof.

The skilled person is well aware that dsRNAs comprising a duplex structure of between 20 and 23, but specifically 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir et al., EMBO 2001, 20:6877-6888). However, others have found that shorter or longer dsRNAs can be effective as well. In

the embodiments described above the dsRNAs of the invention can comprise at least one strand of a length of minimally 21 nt. It can be reasonably expected that shorter dsRNAs comprising a known sequence minus only a few nucleotides on one or both ends may be similarly effective as compared to the dsRNAs of the lengths described above. Hence, dsRNAs comprising a partial sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides, and differing in their ability to inhibit the expression of the target gene by not more than 5, 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence, are contemplated by the invention. Further dsRNAs that cleave within the target sequence can readily be made using the target gene sequence and the target sequence provided.

Double-stranded and single-stranded oligonucleotides that are effective in inducing RNA interference are also referred to as siRNA, RNAi agent and/or iRNA agent. These RNA interference inducing oligonucleotides associate with a cytoplasmic multi-protein complex known as RNAi-induced silencing complex (RISC). In many embodiments, single-stranded and double stranded RNAi agents are sufficiently long that they can be cleaved by an endogenous molecule, e.g. by Dicer, to produce smaller oligonucleotides that can enter the RISC machinery and participate in RISC mediated cleavage of a target sequence, e.g. a target mRNA.

The present invention further includes RNAi agents that target within the sequence targeted by one of the agents of the present invention. As used herein a second RNAi agent is said to target within the sequence of a first RNAi agent if the second RNAi agent cleaves the message anywhere within the mRNA that is complementary to the antisense strand of the first RNAi agent. Such a second agent will generally consist of at least 15 contiguous nucleotides coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in the target gene.

The dsRNA of the invention can contain one or more mismatches to the target sequence. In a preferred embodiment, the dsRNA of the invention contains no more than 3 mismatches. If the antisense strand of the dsRNA contains mismatches to a target sequence, it is preferable that the area of mismatch not be located in the center of the region of complementarity. If the antisense strand of the dsRNA contains mismatches to

the target sequence, it is preferable that the mismatch be restricted to 5 nucleotides from either end, for example 5, 4, 3, 2, or 1 nucleotide from either the 5' or 3' end of the region of complementarity. For example, for a 23 nucleotide dsRNA strand which is complementary to a region of the target gene, the dsRNA generally does not contain any mismatch within the central 13 nucleotides. The methods described within the invention can be used to determine whether a dsRNA containing a mismatch to a target sequence is effective in inhibiting the expression of the target gene. Consideration of the efficacy of dsRNAs with mismatches in inhibiting expression of the target gene is important, especially if the particular region of complementarity in the target gene is known to have polymorphic sequence variation within the population.

In certain embodiment, the sense-strand comprises a mismatch to the antisense strand. In some embodiments, the mismatch is at the 5 nucleotides from the 3'-end, for example 5, 4, 3, 2, or 1 nucleotide from the end of the region of complementarity. In some embodiments, the mismatch is located in the target cleavage site region. In one embodiment, the sense strand comprises no more than 1, 2, 3, 4 or 5 mismatches to the antisense strand. In preferred embodiments, the sense strand comprises no more than 3 mismatches to the antisense strand.

In one embodiment, the sense strand comprises a nucleobase modification, e.g. an optionally substituted natural or non-natural nucleobase, a universal nucleobase, in the target cleavage site region.

The "target cleavage site" herein means the backbone linkage in the target gene, e.g. target mRNA, or the sense strand that is cleaved by the RISC mechanism by utilizing the iRNA agent. And the "target cleavage site region" comprises at least one or at least two nucleotides on both side of the cleavage site. For the sense strand, the target cleavage site is the backbone linkage in the sense strand that would get cleaved if the sense strand itself was the target to be cleaved by the RNAi mechanism. The target cleavage site can be determined using methods known in the art, for example the 5'-RACE assay as detailed in Soutschek *et al., Nature* (2004) 432, 173-178. As is well understood in the art, the cleavage site region for a conical double stranded RNAi agent comprising two 21-nucleotides long strands (wherin the strands form a double stranded

region of 19 consective basepairs having 2-nucleotide single stranded overhangs at the 3'-ends), the cleavage site region corresponds to postions 9-12 from the 5'-end of the sense strand.

In one embodiment, at least one end of the dsRNA has a single-stranded nucleotide overhang of 1 to 4, generally 1 or 2 nucleotides. In one embodiment, the single-stranded overhang has the sequence 5'-GCNN-3', wherein N is independently for each occuurence, A, G, C, U, dT, dU or absent. dsRNAs having at least one nucleotide overhang have unexpectedly superior inhibitory properties than their blunt-ended counterparts. Moreover, the present inventors have discovered that the presence of only one nucleotide overhang strengthens the interference activity of the dsRNA, without affecting its overall stability. dsRNA having only one overhang has proven particularly stable and effective in vivo, as well as in a variety of cells, cell culture mediums, blood, and serum. Generally, the single-stranded overhang is located at the 3'-terminal end of the antisense strand or, alternatively, at the 3'-terminal end of the sense strand. The dsRNA may also have a blunt end, generally located at the 5'-end of the antisense strand. Generally, the antisense strand of the dsRNA has a nucleotide overhang at the 3'-end, and the 5'-end is blunt.

In one embodiment, the antisense strand of the dsRNA has 1-10 nucleotides overhangs each at the 3' end and the 5' end over the sense strand. In one embodiment, the sense strand of the dsRNA has 1-10 nucleotides overhangs each at the 3' end and the 5' end over the antisense strand.

The dsRNAs of the invention may comprise any oligonucleotide modification described herein and below. In certain instances, it may be desirable to modify one or both strands of a dsRNA. In some cases, the two strands will include different modifications. Multiple different modifications can be included on each of the strands. The modifications on a given strand may differ from each other, and may also differ from the various modifications on other strands. For example, one strand may have a modification, e.g., a modification described herein, and a different strand may have a different modification, e.g., a different modification described herein. In other cases, one

strand may have two or more different modifications, and the another strand may include a modification that differs from the at least two modifications on the other strand.

In one embodiment, the dsRNA is chemically modified to enhance stability. In one preferred embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

The present invention also includes dsRNA compounds which are chimeric compounds. "Chimeric" dsRNA compounds or "chimeras," in the context of this invention, are dsRNA compounds, particularly dsRNAs, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an dsRNA compound. These dsRNAs typically contain at least one region wherein the dsRNA is modified so as to confer upon the dsRNA increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the dsRNA may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNAduplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of dsRNA inhibition of gene expression.

The present invention also includes dsRNAs wherein the two strands are linked together. The two strands can be linked together by a polynucleotide linker such as $(dT)_n$; wherein n is 4-10, and thus forming a hairpin. 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 polynucleotide linker.

Hairpin RNAi 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 one embodiment, ranges for the duplex region are 10-35, 12-30, 12-25, 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 one embodiment on the antisense side of the hairpin. In one embodiment, the overhangs are 2-3 nucleotides in length.

The RNAi agents of the invention can target more than one RNA region. For example, an RNAi agent can include a first and second sequence that are sufficiently complementary to each other to hybridize. The first sequence can be complementary to a first target RNA region and the second sequence can be complementary to a second target RNA region. The first and second sequences of the RNAi agent can be on different RNA strands, and the mismatch between the first and second sequences can be less than 50%, 40%, 30%, 20%, 10%, 5%, or 1%. The first and second sequences of the RNAi agent can be on the same RNA strand, and in a related embodiment more than 50%, 60%, 70%, 80%, 90%, 95%, or 1% of the RNAi agent can be in bimolecular form. The first and second sequences of the RNAi agent can be fully complementary to each other.

The first target RNA region can be encoded by a first gene and the second target RNA region can encoded by a second gene, or the first and second target RNA regions can be different regions of an RNA from a single gene. The first and second sequences can differ by at least 1 nucleotide.

The first and second target RNA regions can be on transcripts encoded by first and second sequence variants, e.g., first and second alleles, of a gene. The sequence variants can be mutations, or polymorphisms, for example. The first target RNA region can include a nucleotide substitution, insertion, or deletion relative to the second target RNA region, or the second target RNA region can a mutant or variant of the first target region.

The first and second target RNA regions can comprise viral or human RNA regions. The first and second target RNA regions can also be on variant transcripts of an oncogene or include different mutations of a tumor suppressor gene transcript. In addition, the first and second target RNA regions can correspond to hot-spots for genetic variation.

The double stranded oligonucleotides can be optimized for RNA interference by increasing the propensity of the duplex to disassociate or melt (decreasing the free energy of duplex association), in the region of the 5' end of the antisense strand This can be accomplished, e.g., by the inclusion of modifications or modified nucleosides which increase the propensity of the duplex to disassociate or melt in the region of the 5' end of

the antisense strand. It can also be accomplished by inclusion of modifications or modified nucleosides or attachment of a ligand that increases the propensity of the duplex to disassociate of melt in the region of the 5'end of the antisense strand. While not wishing to be bound by theory, the effect may be due to promoting the effect of an enzyme such as helicase, for example, promoting the effect of the enzyme in the proximity of the 5' end of the antisense strand.

Modifications which increase the tendency of the 5' end of the antisense strand in the duplex to dissociate can be used alone or in combination with other modifications described herein, e.g., with modifications which decrease the tendency of the 3' end of the antisense in the duplex to dissociate. Likewise, modifications which decrease the tendency of the 3' end of the antisense in the duplex to dissociate can be used alone or in combination with other modifications described herein, e.g., with modifications which increase the tendency of the 5' end of the antisense in the duplex to dissociate.

Nucleic acid base pairs 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; I:C is preferred over G:C (I=inosine); mismatches, e.g., non-canonical or other than canonical pairings are preferred over canonical (A:T, A:U, G:C) pairings; pairings which include a universal base are preferred over canonical pairings.

It is preferred that pairings which decrease the propensity to form a duplex are used at 1 or more of the positions in the duplex at the 5' end of the antisense strand. The terminal pair (the most 5' pair in terms of the antisense strand), and the subsequent 4 base pairing positions (going in the 3' direction in terms of the antisense strand) in the duplex are preferred for placement of modifications to decrease the propensity to form a duplex. More preferred are placements in the terminal most pair and the subsequent 3, 2, or 1 base pairings. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the base pairs from the 5'-end of antisense strand in the duplex be chosen independently from the group of: A:U, G:U, I:C, mismatched pairs, e.g., non-canonical or other than canonical

pairings or pairings which include a universal base. In a preferred embodiment at least one, at least 2, or at least 3 base-pairs include a universal base.

Modifications or changes which promote dissociation are preferably made in the sense strand, though in some embodiments, such modifications/changes will be made in the antisense strand.

Nucleic acid base pairs can also be ranked on the basis of their propensity to promote stability and inhibit 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 duplex stability: G:C is preferred over A:U, Watson-Crick matches (A:T, A:U, G:C) are preferred over non-canonical or other than canonical pairings, analogs that increase stability are preferred over Watson-Crick matches (A:T, A:U, G:C), e.g. 2-amino-A:U is preferred over A:U, 2-thio U or 5 Me-thio-U:A, are preferred over U:A, G-clamp (an analog of C having 4 hydrogen bonds):G is preferred over C:G, guanadinium-G-clamp:G is preferred over C:G, psuedo uridine:A, is preferred over U:A, sugar modifications, e.g., 2' modifications, e.g., 2'F, ENA, or LNA, which enhance binding are preferred over non-modified moieties and can be present on one or both strands to enhance stability of the duplex.

It is preferred that pairings which increase the propensity to form a duplex are used at 1 or more of the positions in the duplex at the 3' end of the antisense strand. The terminal pair (the most 3' pair in terms of the antisense strand), and the subsequent 4 base pairing positions (going in the 5' direction in terms of the antisense strand) in the duplex are preferred for placement of modifications to decrease the propensity to form a duplex. More preferred are placements in the terminal most pair and the subsequent 3, 2, or 1 base pairings. It is preferred that at least 1, and more preferably 2, 3, 4, or 5 of the pairs of the recited regions be chosen independently from the group of: G:C, a pair having an analog that increases stability over Watson-Crick matches (A:T, A:U, G:C), 2-amino-A:U, 2-thio U or 5 Me-thio-U:A, G-clamp (an analog of C having 4 hydrogen bonds):G, guanadinium-G-clamp:G, psuedo uridine:A, a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 2'F, ENA, or LNA, which enhance

binding. In some embodiments, at least one, at least, at least 2, or at least 3, of the base pairs promote duplex stability.

In a preferred embodiment the at least one, at least 2, or at least 3, of the base pairs are a pair in which one or both subunits has a sugar modification, e.g., a 2' modification, e.g., 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) and 2'-O-CH_2CH_2-(4'-C) (ENA), which enhances binding.

G-clamps and guanidinium G-clamps are discussed in the following references: Holmes and Gait, "The Synthesis of 2'-O-Methyl G-Clamp Containing Oligonucleotides and Their Inhibition of the HIV-1 Tat-TAR Interaction," Nucleosides, Nucleotides & Nucleic Acids, 22:1259-1262, 2003; Holmes *et al.*, "Steric inhibition of human immunodeficiency virus type-1 Tat-dependent trans-activation in vitro and in cells by oligonucleotides containing 2'-O-methyl G-clamp ribonucleoside analogues," Nucleic Acids Research, 31:2759-2768, 2003; Wilds, *et al.*, "Structural basis for recognition of guanosine by a synthetic tricyclic cytosine analogue: Guanidinium G-clamp," Helvetica Chimica Acta, 86:966-978, 2003; Rajeev, *et al.*, "High-Affinity Peptide Nucleic Acid Oligomers Containing Tricyclic Cytosine Analogues," Organic Letters, 4:4395-4398, 2002; Ausin, *et al.*, "Synthesis of Amino- and Guanidino-G-Clamp PNA Monomers," Organic Letters, 4:4073-4075, 2002; Maier *et al.*, "Nuclease resistance of oligonucleotides containing the tricyclic cytosine analogues phenoxazine and 9-(2-aminoethoxy)-phenoxazine ("G-clamp") and origins of their nuclease resistance properties," Biochemistry, 41:1323-7, 2002; Flanagan, *et al.*, "A cytosine analog that confers enhanced potency to antisense oligonucleotides," Proceedings Of The National Academy Of Sciences Of The United States Of America, 96:3513-8, 1999.

As is discussed above, an oligonucleotide can be modified to both decrease the stability of the antisense 5'end of the duplex and increase the stability of the antisense 3' end of the duplex. This can be effected by combining one or more of the stability decreasing modifications in the antisense 5' end of the duplex with one or more of the stability increasing modifications in the antisense 3' end of the duplex.

Single-stranded oligonucleotides

The single-stranded oligonucleotides of the present invention also comprise nucleotide sequence that is substantially complementary to a "sense" nucleic acid encoding a gene expression product, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an RNA sequence, *e.g.*, a pre-mRNA, mRNA, miRNA, or pre-miRNA. The region of complementarity is less than 30 nucleotides in length, and at least 15 nucleotides in length. Generally, the single stranded oligonucleotides are 10 to 25 nucleotides in length (*e.g.*, 11, 12, 13, 14, 15, 16, 18, 19, 20, 21, 22, 23, or 24 nucleotides in length). In one embodiment the strand is 25-30 nucleotides. Single strands having less than 100% complementarity to the target mRNA, RNA or DNA are also embraced by the present invention. These single-stranded oligonucleotides are also referred to as antisense, antagomir and antimir oligonucleotides.

The single-stranded oligonucleotide can hybridize to a complementary RNA, and prevent access of the translation machinery to the target RNA transcript, thereby preventing protein synthesis. The single-stranded oligonucleotide can also hybridize to a complementary RNA and the RNA target can be subsequently cleaved by an enzyme such as RNase H. Degradation of the target RNA prevents translation.

Single-stranded oligonucleotides, including those described and/or identified as single stranded siRNAs, microRNAs or mirs which may be used as targets or may serve as a template for the design of oligonucleotides of the invention are taught in, for example, Esau, *et al.* US Publication #20050261218 (USSN: 10/909125) entitled "Oligonucleotides and compositions for use in modulation small non-coding RNAs" the entire contents of which is incorporated herein by reference. It will be appreciated by one of skill in the art that any oligonucleotide chemical modifications or variations describe herein also apply to single stranded oligonucleotides.

MicroRNAs (miRNAs or mirs) are a highly conserved class of small RNA molecules that are transcribed from DNA in the genomes of plants and animals, but are not translated into protein. Pre-microRNAs are processed into miRNAs. Processed microRNAs are single stranded ~17-25 nucleotide (nt) RNA molecules that become incorporated into the RNA-induced silencing complex (RISC) and have been identified

as key regulators of development, cell proliferation, apoptosis and differentiation. They are believed to play a role in regulation of gene expression by binding to the 3'-untranslated region of specific mRNAs. RISC mediates down-regulation of gene expression through translational inhibition, transcript cleavage, or both. RISC is also implicated in transcriptional silencing in the nucleus of a wide range of eukaryotes.

The number of miRNA sequences identified to date is large and growing, illustrative examples of which can be found, for example, in: "*miRBase: microRNA sequences, targets and gene nomenclature*" Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. NAR, 2006, 34, Database Issue, D140-D144; "*The microRNA Registry*" Griffiths-Jones S. NAR, 2004, 32, Database Issue, D109-D111; and also on the worldwide web at http://microrna.dot.sanger.dot.ac.dot.uk/sequences/ .

Antagomirs are RNA-like oligonucleotides that harbor various modifications for RNAse protection and pharmacologic properties, such as enhanced tissue and cellular uptake. They differ from normal RNA by, for example, complete 2'-*O*-methylation of sugar, phosphorothioate backbone and, for example, a cholesterol-moiety at 3'-end. Antagomirs may be used to efficiently silence endogenous miRNAs by forming duplexes comprising the antagomir and endogenous miRNA, thereby preventing miRNA-induced gene silencing. An example of antagomir-mediated miRNA silencing is the silencing of miR-122, described in Krutzfeldt *et al*, Nature, 2005, 438: 685-689, which is expressly incorporated by reference herein in its entirety. Antagomir RNAs may be synthesized using standard solid phase oligonucleotide synthesis protocols. See US Patent Application Ser. Nos. 11/502,158 and 11/657,341 (the disclosure of each of which are incorporated herein by reference).

An antagomir can include ligand-conjugated monomer subunits and monomers for oligonucleotide synthesis. Exemplary monomers are described in U.S. Application No. 10/916,185, filed on August 10, 2004. An antagomir can have a ZXY structure, such as is described in PCT Application No. PCT/US2004/07070 filed on March 8, 2004. An antagomir can be complexed with an amphipathic moiety. Exemplary amphipathic moieties for use with oligonucleotide agents are described in PCT Application No. PCT/US2004/07070, filed on March 8, 2004.

Single stranded siRNAs (ss siRNAs) are known and are described in US publication US 2006/0166910 and hereby incorporated by herein by its entirety. Preferably, the single-stranded RNA molecule has a length from 15-29 nucleotides. The RNA-strand may have a 3'hydroxyl group. In some cases, however, it may be preferable to modify the 3' end to make it resistant against 3' to 5' exonucleases. Tolerated 3'-modifications are for example terminal 2'-deoxy nucleotides, 3' phosphate, 2',3'-cyclic phosphate, C3 (or C6, C7, C12) aminolinker, thiol linkers, carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), biotin, fluoresceine, etc. Single stranded siRNAs of the invention include at least one of the following motifs: 5' phosphorothioate or 5'-phosphorodithioate, nucleotides 1 and 2 having cationic modifications via C-5 position of pyrimidines, 2-Position of Purines, N2-G, G-clamp, 8-position of purines, 6-position of purines, internal nucleotides having a 2'-F sugar with base modifications (Pseudouridine, G-clamp, phenoxazine, pyridopyrimidines, gem2'-Me-up/2'-F-down), 3'-end with two purines with novel 2'-substituted MOE analogs, 5'-end nucleotides with novel 2'-substituted MOE analogs, 5'-end having a 3'-F and a 2'-5'-linkage, 4'-substituted nucleoside at the nucleotide 1 at 5'-end and the substituent is cationic, alkyl, alkoxyalkyl, thioether and the like , 4'-substitution at the 3'-end of the strand, and combinations thereof.

Ribozymes are oligonucleotides having specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc Natl Acad Sci U S A. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). At least six basic varieties of naturally-occurring enzymatic RNAs are known presently. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved

its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

Methods of producing a ribozyme targeted to any target sequence are known in the art. Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, each specifically incorporated herein by reference, and synthesized to be tested *in vitro* and *in vivo*, as described therein.

Aptamers are nucleic acid or peptide molecules that bind to a particular molecule of interest with high affinity and specificity (Tuerk and Gold, Science **249**:505 (1990); Ellington and Szostak, Nature **346**:818 (1990)). DNA or RNA aptamers have been successfully produced which bind many different entities from large proteins to small organic molecules. See Eaton, Curr. Opin. Chem. Biol. **1**:10-16 (1997), Famulok, Curr. Opin. Struct. Biol. **9**:324-9(1999), and Hermann and Patel, Science **287**:820-5 (2000). Aptamers may be RNA or DNA based. Generally, aptamers are engineered through repeated rounds of *in vitro* selection or equivalently, SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms. The aptamer may be prepared by any known method, including synthetic, recombinant, and purification methods, and may be used alone or in combination with other aptamers specific for the same target. Further, as described more fully herein, the term "aptamer" specifically includes "secondary aptamers" containing a consensus sequence derived from comparing two or more known aptamers to a given target.

Immunostimulatory Oligonucleotides

Nucleic acids of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal or other patient. The immune response may be an innate or an adaptive immune response. The immune system is divided into a more innate immune system, and acquired adaptive immune system of vertebrates, the latter of which is further divided into humoral cellular components. In particular embodiments, the immune response may be mucosal.

Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target polynucleotide in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a seuqence correspondign to a region of a naturally occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in said CpG dinucleotide is methylated. Methods of immune stimulation using single stranded oligonucleotides and immune stimulatory oligonucleotides.

The immunostimulatory nucleic acid or oligonucleotide comprises capable of inducing an anti-viral or an antibacterial response, in particular, the induction of type I IFN, IL-18 and/or IL-1β by modulating RIG-I.

Other oligonucleotides

Because transcription factors recognize their relatively short binding sequences, even in the absence of surrounding genomic DNA, short oligonucleotides bearing the consensus binding sequence of a specific transcription factor can be used as tools for manipulating gene expression in living cells. This strategy involves the intracellular delivery of such "decoy oligonucleotides", which are then recognized and bound by the target factor. Occupation of the transcription factor's DNA-binding site by the decoy renders the transcription factor incapable of subsequently binding to the promoter regions of target genes. Decoys can be used as therapeutic agents, either to inhibit the expression of genes that are activated by a transcription factor, or to upregulate genes that are suppressed by the binding of a transcription factor. Examples of the utilization of decoy

oligonucleotides may be found in Mann et al., J. Clin. Invest., 2000, 106: 1071-1075, which is expressly incorporated by reference herein, in its entirety.

U1 adaptor inhibit polyA sites and are bifunctional oligonucleotides with a target domain complementary to a site in the target gene's terminal exon and a 'U1 domain' that binds to the U1 smaller nuclear RNA component of the U1 snRNP (Goraczniak, et al., 2008, Nature Biotechnology, 27(3), 257-263, which is expressly incorporated by reference herein, in its entirety). U1 snRNP is a ribonucleoprotein complex that functions primarily to direct early steps in spliceosome formation by binding to the pre-mRNA exon- intron boundary (Brown and Simpson, 1998, Annu Rev Plant Physiol Plant MoI Biol 49:77-95). Nucleotides 2-11 of the 5'end of U1 snRNA base pair bind with the 5'ss of the pre mRNA. In one embodiment, oligonucleotides of the invention are U1 adaptors. In one embodiment, the U1 adaptor can be administered in combination with at least one other iRNA agent.

Oligonucleotide modifications

Unmodified oligonucleotides may be less than optimal in some applications, *e.g.*, unmodified oligonucleotides can be prone to degradation by *e.g.*, cellular nucleases. Nucleases can hydrolyze nucleic acid phosphodiester bonds. However, chemical modifications to one or more of the above oligonucleotide components can confer improved properties, and, *e.g.*, can render oligonucleotides more stable to nucleases.

Modified nucleic acids and nucleotide surrogates can include one or more of:

(i) alteration, *e.g.*, replacement, of one or both of the non-linking phosphate oxygens and/or of one or more of the linking phosphate oxygens in the phosphodiester backbone linakge.

(ii) alteration, *e.g.*, replacement, of a constituent of the ribose sugar, *e.g.*, of the 2' hydroxyl on the ribose sugar;

(iii) wholesale replacement of the phosphate moiety with "dephospho" linkers;

(iv) modification or replacement of a naturally occurring base with a non-natural base;

(v) replacement or modification of the ribose-phosphate backbone;

(vi) modification of the 3' end or 5' end of the oligonucelotide, *e.g.*, removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, *e.g.,* a fluorescently labeled moiety, to either the 3'' or 5' end of oligonucleotide; and

(vii) modification of the sugar (e.g., six membered rings).

The terms replacement, modification, alteration, and the like, as used in this context, do not imply any process limitation, *e.g.*, modification does not mean that one must start with a reference or naturally occurring ribonucleic acid and modify it to produce a modified ribonucleic acid bur rather modified simply indicates a difference from a naturally occurring molecule.

As oligonucleotides are polymers of subunits or monomers, many of the modifications described herein can occur at a position which is repeated within an oligonucleotide, *e.g.*, a modification of a nucleobase, a sugar, a phosphate moiety, or the non-bridging oxygen of a phosphate moiety. It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at a single nucleoside within an oligonucleotide.

In some cases the modification will occur at all of the subject positions in the oligonucleotide but in many, and in fact in most cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in the internal region, may only occur in a terminal regions, *e.g.* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of an oligonucleotide. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of an oligonucleotide or may only occur in a single strand region of an oligonucleotide. *E.g.*, a phosphorothioate modification at a non-bridging oxygen position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

A modification described herein may be the sole modification, or the sole type of modification included on multiple nucleotides, or a modification can be combined with one or more other modifications described herein. The modifications described herein can also be combined onto an oligonucleotide, e.g. different nucleotides of an oligonucleotide have different modifications described herein.

In some embodiments it is particularly preferred, *e.g.*, to enhance stability, to include particular nucleobases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang will be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' OH group of the ribose sugar, *e.g.*, the use of deoxyribonucleotides, *e.g.*, deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, *e.g.*, phosphothioate modifications. Overhangs need not be homologous with the target sequence.

Specific modifications are discussed in more detail below.

The Phosphate Group

The phosphate group is a negatively charged species. The charge is distributed equally over the two non-bridging oxygen atoms. However, the phosphate group can be modified by replacing one of the oxygens with a different substituent. One result of this modification to RNA phosphate backbones can be increased resistance of the oligoribonucleotide to nucleolytic breakdown. Thus while not wishing to be bound by theory, it can be desirable in some embodiments to introduce alterations which result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. In one embodiment, one of the non-bridging phosphate oxygen atoms in the phosphate backbone moiety 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, alkyl, aryl), or OR (R is 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).

Phosphorodithioates have both non-bridging oxygens replaced by sulfur. The phosphorus center in the phosphorodithioates is achiral which precludes the formation of oligoribonucleotides 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, may be desirable in that they cannot produce diastereomer mixtures. Thus, the non-bridging oxygens can be independently any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl).

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

Replacement of the Phosphate Group

The phosphate group can be replaced by non-phosphorus containing connectors. 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.

Examples of moieties which can replace the phosphate group include methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methylenecarbonylamino and methylenemethylimino groups.

Modified phosphate linkages where at least one of the oxygens 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 backbone linkage."

Replacement of Ribophosphate Backbone

Oligonucleotide- mimicking scaffolds can also be constructed wherein the phosphate linker and ribose 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 mophilino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates. A preferred surrogate is a PNA surrogate.

Sugar modifications

An oligonucleotide can include modification of all or some of the sugar groups of the nucleic acid. *E.g.*, the 2′ hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents. While not being bound by theory, enhanced stability is expected since the hydroxyl can no longer be deprotonated to form a 2′-alkoxide ion. The 2′-alkoxide can catalyze degradation by intramolecular nucleophilic attack on the linker phosphorus atom. Again, while not wishing to be bound by theory, it can be desirable to some embodiments to introduce alterations in which alkoxide formation at the 2′ position is not possible.

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), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2′ hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4′ carbon of the same ribose sugar; O-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy, $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), ($OCH_2CH_2OCH_3$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

"Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); 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; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality.

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 nucleotides 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. Oligonucleotides can also include "abasic" sugars, which lack a nucleobase at C-1′. These abasic sugars can also be further containing modifications at one or more of the constituent sugar atoms. Oligonucleotides can also contain one or more sugars that are in the L form, e.g. L-nucleosides.

Preferred substitutents are 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) and 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE).

Terminal Modifications

The 3′ and 5′ ends of an oligonucleotide can be modified. Such modifications can be at the 3′ end, 5′ end or both ends of the molecule. They can include modification or replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. *E.g.*, the 3′ and 5′ ends of an oligonucleotide 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).

When a linker/phosphate-functional molecular entity-linker/phosphate array is interposed between two strands of a dsRNA, this array can substitute for a hairpin RNA loop in a hairpin-type RNA agent.

Terminal modifications useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. *E.g.*, in preferred embodiments antisense strands of dsRNAs, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 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. Suitable modifications include: 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'-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'); 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'); any additional combination of oxgen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-beta-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)2(O)P-NH-5', (HO)(NH2)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5'-, (OH)2(O)P-5'-CH2-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH2-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-). Other embodiments include replacement of oxygen/sulfur with BH_3, BH_3^- and/or Se.

Terminal modifications can also be useful for monitoring distribution, and in such cases the preferred groups to be added include fluorophores, *e.g.*, fluorscein or an Alexa dye, *e.g.*, Alexa 488. Terminal modifications can also be useful for enhancing uptake, useful modifications for this include cholesterol. Terminal modifications can also be useful for cross-linking an RNA agent to another moiety; modifications useful for this include mitomycin C.

End-caps for exonuclease protection

Nucleobases

Adenine, guanine, cytosine and uracil are the most common bases found in RNA. These bases can be modified or replaced to provide RNA's having improved properties. For example, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases and "universal bases" can be employed. Examples include 2-(halo)adenine, 2-(alkyl)adenine, 2-(propyl)adenine, 2-(amino)adenine, 2-(aminoalkyll)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, or any O-alkylated or N-alkylated derivatives thereof;

Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, hereby incorporated by refernence, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed.

John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

Cationic Groups

Modifications to oligonucleotides can also include attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone moiety. A cationic group can be attached to any atom capable of substitution on a natural, unusual or universal base. A preferred position is one that does not interfere with hybridization, i.e., does not interfere with the hydrogen bonding interactions needed for base pairing. A cationic group can be attached e.g., through the C2' position of a sugar or analogous position in a cyclic or acyclic sugar surrogate. Cationic groups can include e.g., 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); or $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino).

End-caps for exonuclease protection

Placement within an oligonucleotide

Some modifications may preferably be included on an oligonucleotide at a particular location, e.g., at an internal position of a strand, or on the 5' or 3' end of an oligonucleotide. A preferred location of a modification on an oligonucleotide, may confer preferred properties on the agent. For example, preferred locations of particular modifications may confer optimum gene silencing properties, or increased resistance to endonuclease or exonuclease activity.

One or more nucleotides of an oligonucleotide may have a 2'-5' linkage. One or more nucleotides of an oligonucleotide may have inverted linkages, e.g. 3'-3', 5'-5', 2'-2' or 2'-3' linkages.

A double-stranded oligonucleotide may include at least one 5'-uridine-adenine-3' (5'-UA-3') dinucleotide wherein the uridine is a 2'-modified nucleotide, or a terminal 5'-uridine-guanine-3' (5'-UG-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a terminal 5'-cytidine-adenine-3' (5'-CA-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a terminal 5'-uridine-uridine-3' (5'-UU-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a terminal 5'-cytidine-cytidine-3' (5'-CC-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a terminal 5'-cytidine-uridine-3' (5'-CU-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a terminal 5'-uridine-cytidine-3' (5'-UC-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide. Double-stranded oligonucleotides including these modifications are particularly stabilized against endonuclease activity.

General References

The oligoribonucleotides and oligoribonucleosides used in accordance with this invention may be synthesized with solid phase synthesis, see for example "Oligonucleotide synthesis, a practical approach", Ed. M. J. Gait, IRL Press, 1984; "Oligonucleotides and Analogues, A Practical Approach", Ed. F. Eckstein, IRL Press, 1991 (especially Chapter 1, Modern machine-aided methods of oligodeoxyribonucleotide synthesis, Chapter 2, Oligoribonucleotide synthesis, Chapter 3, 2'-O--Methyloligoribonucleotide- s: synthesis and applications, Chapter 4, Phosphorothioate oligonucleotides, Chapter 5, Synthesis of oligonucleotide phosphorodithioates, Chapter 6, Synthesis of oligo-2'-deoxyribonucleoside methylphosphonates, and. Chapter 7, Oligodeoxynucleotides containing modified bases. Other particularly useful synthetic procedures, reagents, blocking groups and reaction conditions are described in Martin, P., *Helv. Chim. Acta*, **1995**, *78*, 486-504; Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1992**, *48*, 2223-2311 and Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1993**, *49*, 6123-6194, or

references referred to therein. Modification described in WO 00/44895, WO01/75164, or WO02/44321 can be used herein. The disclosure of all publications, patents, and published patent applications listed herein are hereby incorporated by reference.

Phosphate Group References

The preparation of phosphinate oligoribonucleotides is described in U.S. Pat. No. 5,508,270. The preparation of alkyl phosphonate oligoribonucleotides is described in U.S. Pat. No. 4,469,863. The preparation of phosphoramidite oligoribonucleotides is described in U.S. Pat. No. 5,256,775 or U.S. Pat. No. 5,366,878. The preparation of phosphotriester oligoribonucleotides is described in U.S. Pat. No. 5,023,243. The preparation of borano phosphate oligoribonucleotide is described in U.S. Pat. Nos. 5,130,302 and 5,177,198. The preparation of 3'-Deoxy-3'-amino phosphoramidate oligoribonucleotides is described in U.S. Pat. No. 5,476,925. 3'-Deoxy-3'-methylenephosphonate oligoribonucleotides is described in An, H, *et al. J. Org. Chem.* **2001**, *66*, 2789-2801. Preparation of sulfur bridged nucleotides is described in Sproat *et al. Nucleosides Nucleotides* **1988**, *7*,651 and Crosstick *et al. Tetrahedron Lett.* **1989**, *30*, 4693.

Sugar Group References

Modifications to the 2' modifications can be found in Verma, S. *et al. Annu. Rev. Biochem.* **1998**, *67*, 99-134 and all references therein. Specific modifications to the ribose can be found in the following references: 2'-fluoro (Kawasaki et. al., *J. Med. Chem.*, **1993**, *36*, 831-841), 2'-MOE (Martin, P. *Helv. Chim. Acta* **1996**, *79*, 1930-1938), "LNA" (Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301-310).

Replacement of the Phosphate Group References

Methylenemethylimino linked oligoribonucleosides, also identified herein as MMI linked oligoribonucleosides, methylenedimethylhydrazo linked oligoribonucleosides, also identified herein as MDH linked oligoribonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified herein as amide-3

linked oligoribonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified herein as amide-4 linked oligoribonucleosides as well as mixed backbone compounds having, as for instance, alternating MMI and PO or PS linkages can be prepared as is described in U.S. Pat. Nos. 5,378,825, 5,386,023, 5,489,677 and in published PCT applications PCT/US92/04294 and PCT/US92/04305 (published as WO 92/20822 WO and 92/20823, respectively). Formacetal and thioformacetal linked oligoribonucleosides can be prepared as is described in U.S. Pat. Nos. 5,264,562 and 5,264,564. Ethylene oxide linked oligoribonucleosides can be prepared as is described in U.S. Pat. No. 5,223,618. Siloxane replacements are described in Cormier,J.F. *et al. Nucleic Acids Res.* **1988**, *16*, 4583. Carbonate replacements are described in Tittensor, J.R. *J. Chem. Soc. C* **1971**, 1933. Carboxymethyl replacements are described in Edge, M.D. *et al. J. Chem. Soc. Perkin Trans. 1* **1972**, 1991. Carbamate replacements are described in Stirchak, E.P. Nucleic Acids Res. 1989, 17, 6129.

Replacement of the Phosphate-Ribose Backbone References

Cyclobutyl sugar surrogate compounds can be prepared as is described in U.S. Pat. No. 5,359,044. Pyrrolidine sugar surrogate can be prepared as is described in U.S. Pat. No. 5,519,134. Morpholino sugar surrogates can be prepared as is described in U.S. Pat. Nos. 5,142,047 and 5,235,033, and other related patent disclosures. Peptide Nucleic Acids (PNAs) are known per se and can be prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, Bioorganic & Medicinal Chemistry, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Pat. No. 5,539,083.

Terminal Modification References

Terminal modifications are described in Manoharan, M. *et al. Antisense and Nucleic Acid Drug Development 12*, 103-128 (2002) and references therein.

Bases References

N-2 substitued purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,459,255. 3-Deaza purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,457,191. 5,6-Substituted pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,614,617. 5-Propynyl pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,484,908. Additional references are disclosed in the above section on base modifications

Oligonucleotide production

The oligonucleotide compounds of the invention can be prepared using solution-phase or solid-phase organic synthesis. Organic synthesis offers the advantage that the oligonucleotide strands comprising non-natural or modified nucleotides can be easily prepared. Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides, such as the phosphorothioates, phosphorodithioates and alkylated derivatives. The double-stranded oligonucleotide compounds of the invention may be prepared using a two-step procedure. First, the individual strands of the double-stranded molecule are prepared separately. Then, the component strands are annealed.

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

Teachings regarding the synthesis of particular modified oligonucleotides may be found in the following U.S. patents or pending patent applications: U.S. Pat. Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Pat. No. 5,212,295, drawn to compounds for the preparation of oligonucleotides having chiral phosphorus linkages; U.S. Pat. Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Pat. No. 5,386,023, drawn to backbone-modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Pat. No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and

methods of synthesis thereof; U.S. Pat. No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Pat. No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Pat. No. 5,539,082, drawn to peptide nucleic acids; U.S. Pat. No. 5,554,746, drawn to oligonucleotides having β-lactam backbones; U.S. Pat. No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Pat. No. 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Pat. Nos. 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Pat. No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Pat. No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Pat. No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Pat. No. 5,223,168, and U.S. Pat. No. 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Pat. Nos. 5,602,240, and 5,610,289, drawn to backbone-modified oligonucleotide analogs; and U.S. Pat. Nos. 6,262,241, and 5,459,255, drawn to, inter alia, methods of synthesizing 2'-fluoro-oligonucleotides.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one type of modification may be incorporated in a single oligonucleotide compound or even in a single nucleotide thereof.

Methods for inhibiting expression of a target gene

In yet another aspect, the invention relates to a method for inhibiting the expression of a target gene in a cell or organism. In one embodiment, the method includes administering the inventive oligonucleotide, e.g. antisense, aptamer, antagomir, or an iRNA agent; or a pharmaceutical composition containing the said oligonucleotide to a cell or an organism, such as a mammal, such that expression of the target gene is silenced. Compositions and methods for inhibiting the expression of a target gene using the inventive oligonucleotide, e.g. an iRNA agent, can be performed as described in the preceding sections.

In this embodiment, a pharmaceutical composition containing the inventive oligonucleotide may be administered by any means known in the art including, but not limited to oral or parenteral routes, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), ocular, rectal, vaginal, and topical (including buccal and sublingual) administration. In preferred embodiments, the pharmaceutical compositions are administered by intravenous or intraparenteral infusion or injection. The pharmaceutical compositions can also be administered intraparenchymally, intrathecally, and/or by stereotactic injection.

The methods for inhibiting the expression of a target gene can be applied to any gene one wishes to silence, thereby specifically inhibiting its expression, provided the cell or organism in which the target gene is expressed includes the cellular machinery which effects RNA interference. Examples of genes which can be targeted for silencing include, without limitation, developmental genes including but not limited to adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, and neurotransmitters and their receptors; (2) oncogenes including but not limited to ABLI, BCL1, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3 and YES; (3) tumor suppresser genes including but not limited to APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53 and WT1; and (4) enzymes including but not limited to ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, GTPases, helicases, hemicellulases, integrases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, polygalacturonases, proteinases and peptideases, pullanases, recombinases, reverse transcriptases, topoisomerases, and xylanases.

In addition to in vivo gene inhibition, the skilled artisan will appreciate that the inventive oligonucleotides, e.g. iRNA agent, of the present invention are useful in a wide variety of *in vitro* applications. Such *in vitro* applications, include, for example, scientific and commercial research (e.g., elucidation of physiological pathways, drug discovery and development), and medical and veterinary diagnostics. In general, the method involves the introduction of the oligonucleotide, e.g. an iRNA agent, into a cell using known techniques (e.g., absorption through cellular processes, or by auxiliary agents or devices, such as electroporation and lipofection), then maintaining the cell for a time sufficient to obtain degradation of an mRNA transcript of the target gene.

Definitions

The term "nucleoside" includes nucleotides as well as nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

The term "aliphatic," as used herein, refers to a straight or branched hydrocarbon radical containing up to twenty four carbon atoms wherein the saturation between any two carbon atoms is a single, double or triple bond. An aliphatic group preferably contains from 1 to about 24 carbon atoms, more typically from 1 to about 12 carbon atoms. 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. The straight or branched chain of an aliphatic group may be interrupted with one or more heteroatoms that include nitrogen, oxygen, sulfur and phosphorus. Such aliphatic groups interrupted by heteroatoms include without limitation polyalkoxys, such as polyalkylene glycols, polyamines, and polyimines, for example. Aliphatic groups as used herein may optionally include further substitutent groups.

The term “alkyl” refers to saturated and unsaturated non-aromatic hydrocarbon chains that may be a straight chain or branched chain, containing the indicated number of

carbon atoms (these include without limitation propyl, allyl, or propargyl), which may be optionally inserted with N, O, or S. For example, C_1-C_{20} indicates that the group may have from 1 to 20 (inclusive) carbon atoms in it. The term "alkoxy" refers to an -O-alkyl radical. The term "alkylene" refers to a divalent alkyl (*i.e.*, -R-). The term "alkylenedioxo" refers to a divalent species of the structure -O-R-O-, in which R represents an alkylene. 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.

The term "cyclic " as used herein includes a cycloalkyl group and a heterocyclic group. Any suitable ring position of the cyclic group may be covalently linked to the defined chemical structure.

The term "acyclic" may describe any carrier that is branched or unbranched, and does not form a closed ring.

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. Examples of aryl groups include phenyl, naphthyl 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.

The term "cycloalkyl" as employed herein includes saturated and partially unsaturated 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, decalin, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

The term "heteroaryl" 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. Examples of heteroaryl groups include pyridyl,

furyl or furanyl, imidazolyl, benzimidazolyl, pyrimidinyl, thiophenyl or thienyl, quinolinyl, indolyl, thiazolyl, 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.

The term "heterocycloalkyl" and "heterocyclic" can be used interchangeably and refer to a non-aromatic 3-, 4-, 5-, 6- or 7-membered ring or a bi- or tri-cyclic group fused system, where (i) each ring contains between one and three heteroatoms independently selected from oxygen, sulfur and nitrogen, (ii) each 5-membered ring has 0 to 1 double bonds and each 6-membered ring has 0 to 2 double bonds, (iii) the nitrogen and sulfur heteroatoms may optionally be oxidized, (iv) the nitrogen heteroatom may optionally be quaternized, (iv) any of the above rings may be fused to a benzene ring, and (v) the remaining ring atoms are carbon atoms which may be optionally oxo-substituted. Representative heterocycloalkyl groups include, but are not limited to, [1,3]dioxolane, pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, and tetrahydrofuryl. Such heterocyclic groups may be further substituted to give substituted heterocyclic.

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.

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

The term "silyl" as used herein is represented by the formula $-SiA^1A^2A^3$, where A^1, A^2, and A^3 can be, independently, hydrogen or a substituted or unsubstituted alkyl, cycloalkyl, alkoxy, alkenyl, cycloalkenyl, alkynyl, cycloalkynyl, aryl, or heteroaryl group as described herein.

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 may be further substituted.

The Bases

Adenine, guanine, cytosine and uracil are the most common bases found in RNA. These bases can be modified or replaced to provide RNA's having improved properties. *E.g.*, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases and "universal bases" can be employed. Examples include 2-aminoadenine, 2-fluoroadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine,7-deazaadenine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4-acetyl cytosine, 2-thiocytosine, N6-methyladenine,

N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases. Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

The term "non-natural" nucleobase refers any one of the following: 2-methyladeninyl, N6-methyladeninyl, 2-methylthio-N6-methyladeninyl, N6-isopentenyladeninyl, 2-methylthio-N6-isopentenyladeninyl, N6-(cis-hydroxyisopentenyl)adeninyl, 2-methylthio-N6-(cis-hydroxyisopentenyl) adeninyl, N6-glycinylcarbamoyladeninyl, N6-threonylcarbamoyladeninyl, 2-methylthio-N6-threonyl carbamoyladeninyl, N6-methyl-N6-threonylcarbamoyladeninyl, N6-hydroxynorvalylcarbamoyladeninyl, 2-methylthio-N6-hydroxynorvalyl carbamoyladeninyl, N6,N6-dimethyladeninyl, 3-methylcytosinyl, 5-methylcytosinyl, 2-thiocytosinyl, 5-formylcytosinyl, N4-methylcytosinyl, 5-hydroxymethylcytosinyl, 1-methylguaninyl, N2-methylguaninyl, 7-methylguaninyl, N2,N2-dimethylguaninyl, N2,7-dimethylguaninyl, N2,N2,7-trimethylguaninyl, 1-methylguaninyl, 7-cyano-7-deazaguaninyl, 7-aminomethyl-7-deazaguaninyl, pseudouracilyl, dihydrouracilyl, 5-methyluracilyl, 1-methylpseudouracilyl, 2-thiouracilyl, 4-thiouracilyl, 2-thiothyminyl 5-methyl-2-thiouracilyl, 3-(3-amino-3-carboxypropyl)uracilyl, 5-hydroxyuracilyl, 5-methoxyuracilyl, uracilyl 5-oxyacetic acid, uracilyl 5-oxyacetic acid methyl ester, 5-(carboxyhydroxymethyl)uracilyl, 5-(carboxyhydroxymethyl)uracilyl methyl ester, 5-methoxycarbonylmethyluracilyl, 5-methoxycarbonylmethyl-2-thiouracilyl, 5-aminomethyl-2-thiouracilyl, 5-methylaminomethyluracilyl, 5-methylaminomethyl-2-thiouracilyl, 5-methylaminomethyl-2-selenouracilyl, 5-carbamoylmethyluracilyl, 5-carboxymethylaminomethyluracilyl, 5-carboxymethylaminomethyl-2-thiouracilyl, 3-methyluracilyl, 1-methyl-3-(3-amino-3-carboxypropyl) pseudouracilyl, 5-carboxymethyluracilyl, 5-methyldihydrouracilyl, 3-methylpseudouracilyl,

EQUIVALENTS

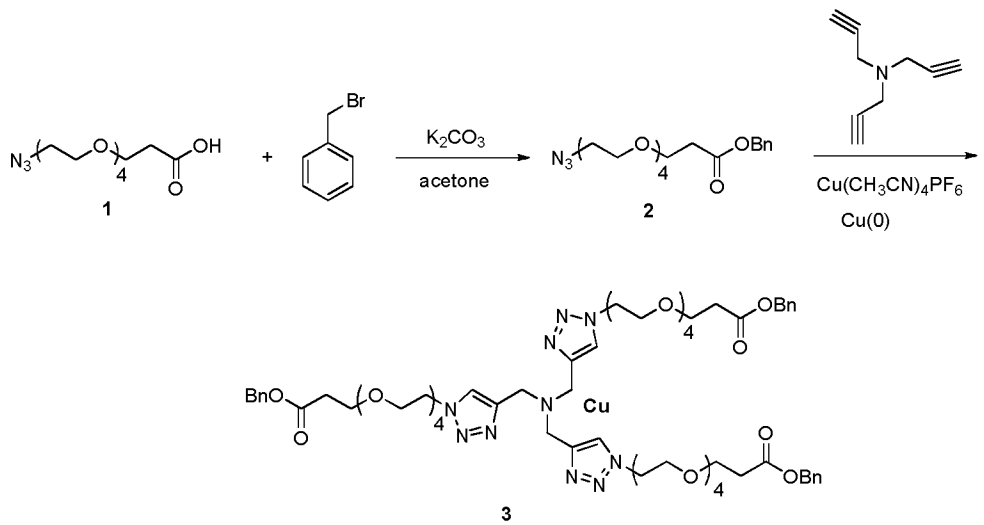
Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed.

EXAMPLES

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 invention, and are not intended to limit the invention. Thus, the invention should in way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1. Design and Synthesis of Novel Solid Supported Cu-Ligand for Click-Chemistry

Scheme I:



Preparation of Compound 2: To a solution of azido-PEG acid (**1**, 2.0 g, 6.88 mmol, 1.0 eq) and benzyl bromide (1.76 g, 10.32 mmol, 1.5 eq) in acetone was added K_2CO_3 (2.84 g, 20.64 mmol, 3.0 eq) as solid and the reaction was refluxed for overnight.

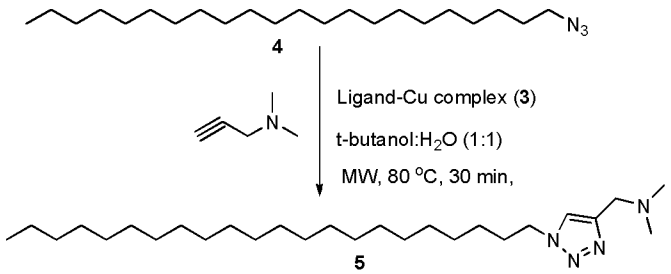
After completion of the reaction by TLC, was cooled to room temperature, filtered K_2CO_3, evaporated the solvent, re-dissolved in the ethylacetate, washed with water. Organic layer was separated, dried on Na_2SO_4, concentrated and purified on column chromatography using hexane and ethylacetate (50%) as gradients to get pure benzyl protected PEG azide (**3**) as an oil in 70% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.22 (m, 5H), 5.13 (s, 2H), 3.77 (t, J = 6.5, 2H), 3.71 – 3.53 (m, 14H), 3.37 (t, J = 5.1, 2H), 2.65 (t, J = 6.5, 2H); calc. mass for $C_{18}H_{27}N_3O_6$ is 381.4; found 404.2 (+Na).

Preparation of compound 3: To a solution of compound (**2**, 0.85 g, 2.25 mmol, 3.0 eq) and tris propargylamine (0.09 g, 0.75 mmol, 1.0 eq) in 4:1 mixture of dichloromethane:methanol at room temperature were added tetrakis methylcyano phosphorous hexaflouro copper (I)complex (0.16 g, 0.45 mmol, 0.6 eq) and elemental Cu(0) (0.02 g, 0.45 mmol, 0.6 eq). Reaction was continued stirring at room temperature for overnight, after completion of the reaction, solvent was evaporated and re-dissolved in the dichloromethane, washed with water. Combined organic layers were dried over $MgSO_4$, filtered, concentrated and purified on the column chromatography using dichloromethane and methanol (10%) as gradients to get pure triazolyl ligand (**3**) as copper complex.

Calc. mass for $C_{63}H_{90}CuN_{10}O_{18}$ is 1338.9; found 1339.5.

Example 2. Click Reaction using Cu-Complex 3:



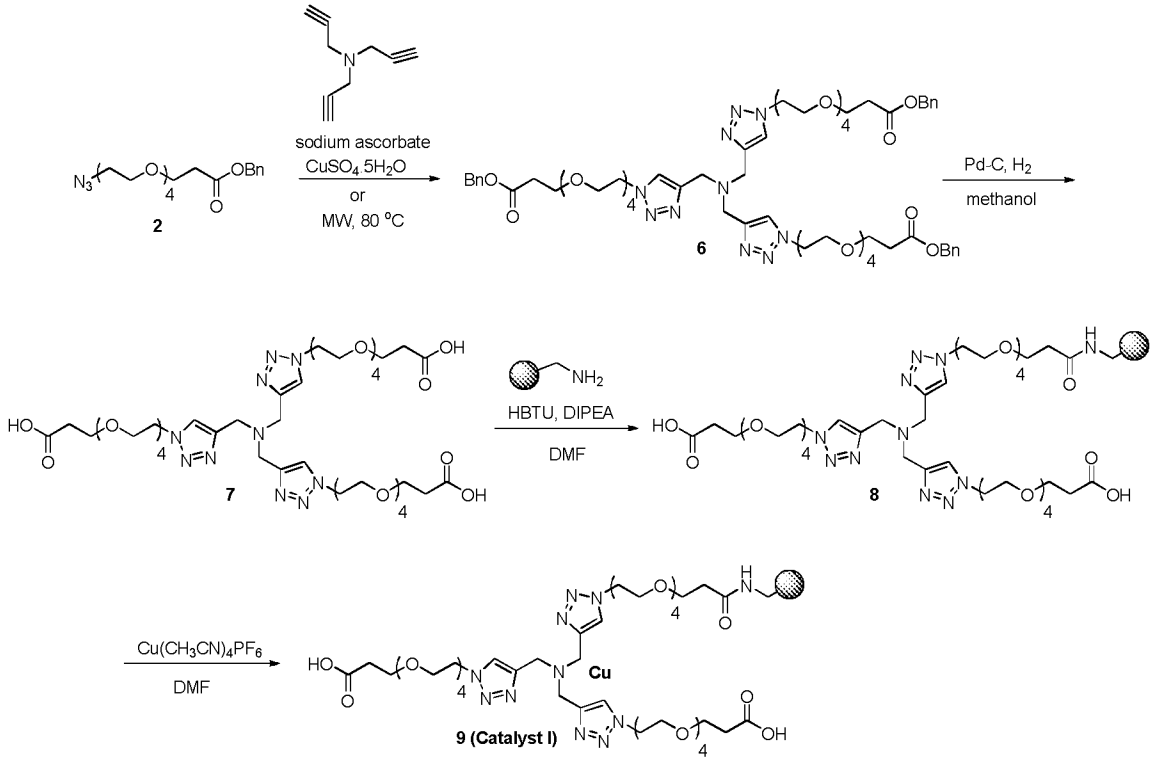
Preparation of compound 5: To a mixture of azide (**4**, 0.07 g, 0.2 mmol, 1.0 eq) and *N*,*N*-dimethyl propargylamine (0.016 g, 0.2 mmol, 1.0 eq) in t-butanol:water (1:1) was added tris-(PEG triazolyl) Cu-complex (**3**, 0.07 g, 0.07 mmol, 0.3 eq) and the

reaction was continued under microwave conditions at 80 °C, and complete consumption of the starting materials were observed after 30 minutes.

Calc. mass for $C_{27}H_{54}N_4$ is 434.74; found 435.4.

Example 3.

Scheme II: Preparation of immobilized Cu Catalyst I (9)



Preparation of Compound 6:

Method A: To a mixture of PEG-azide (**2**, 0.7 g, 1.83 mmol, 3.0 eq) and tris-propargylamine (0.08 g, 0.61 mmol, 1.0 eq) in t-butanol:water (1:1) were added sodium ascorbate (0.036 g, 0.18 mmol, 0.3 eq) followed by $CuSO_4.5H_2O$ (0.004g, 0.018 mmol, 0.03 eq) at room temperature and the reaction was continued for overnight. TLC shows complete consumption of the starting materials, reaction mixture was diluted with excess amount of dichloromethane, separated the organic layer and washed with water. Combined organics were dried on $MgSO_4$, concentrated, purified on column chromatography using dichloromethane and methanol (10%) as gradients to get pure benzyl protected triazolyl ligand (**6**) as an oil in 88% yield.

Method B: To a solution of PEG-azide (**2**, 0.28 g, 0.75 mmol, 3.0 eq) and tris-propargylamine (0.032 g, 0.25 mmol, 1.0 eq) in t-butanol:water (1:1) were added sodium ascorbate (0.015 g, 0.075 mmol, 0.3 eq) followed by $CuSO_4.5H_2O$ (0.0018g, 0.0075

mmol, 0.03 eq) and the reaction was continued under microwave conditions at 80 °C, TLC and LC Mass shows complete consumption of the starting materials after 1h. Reaction mixture was diluted with the dichloromethane, separated the organic layer and washed with water. Combined organics were dried on $MgSO_4$, concentrated, purified on column chromatography using dichloromethane and methanol (10%) as gradients to get pure benzyl protected triazolyl ligand (**6**) as an oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 (s, 3H), 7.41 – 7.19 (m, 15H), 5.12 (s, 6H), 4.52 (t, *J* = 5.3, 6H), 3.87 (t, *J* = 5.3, 6H), 3.76 (dd, *J* = 7.7, 5.2, 12H), 3.60 (d, *J* = 3.4, 36H), 2.64 (t, *J* = 6.5, 6H). Calc. mass for $C_{63}H_{90}N_{10}O_{18}$ is 1274.6; found 1275.5.

Preparation of Compound 7: To a solution of benzyl protected ligand (**6**, 0.6g, 0.47 mmol) in methanol was added catalytic amount of 10% Pd-C, removed air and purged with the argon, repeated the cycles two times and continued the reaction at room temperature under H_2 atmosphere for overnight. After completion of the reaction was filtered through small bed of celite, washed with methanol, combined washings were concentrated and further dried on vacuum to get pure ligand (**7**) as oil in 97% yields.

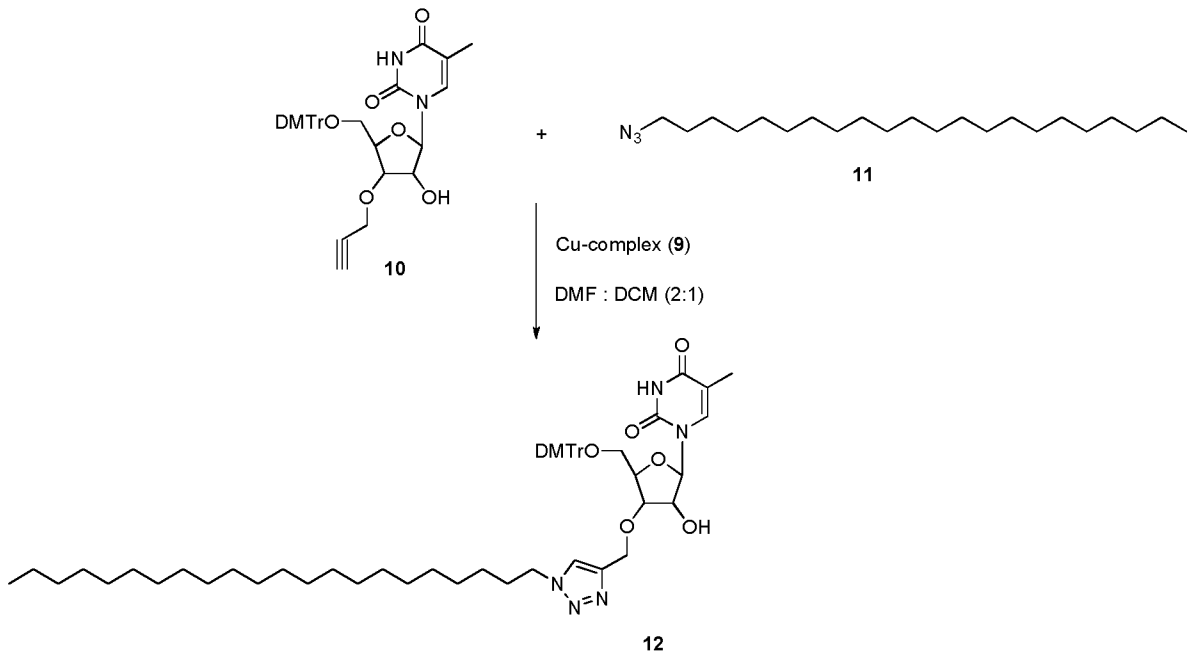
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 3H), 4.57 (t, *J* = 5.1, 6H), 3.99 (s, 5H), 3.90 (t, *J* = 5.1, 6H), 3.75 (t, *J* = 6.1, 6H), 3.65 – 3.54 (m, 35H), 3.47 (s, 5H), 2.56 (t, *J* = 6.1, 6H). Calc. mass for $C_{42}H_{72}N_{10}O_{18}$ is 1004.5; found 1005.3

Preparation of solid supported ligand (8): To a solution of compound 7 (0.88 g, 0.87 mmol), HBTU (0.16 g, 0.43 mmol), DIPEA (0.24 mL, 1.13 mmol) in DMF was added aminomethyl polystyrene resin (230 μmmol/g, 1.52 g, 0.35 mmol) and the mixture was continued on shaker at room temperature for overnight. Filtered the solvent, washed the resin with DCM: methanol (9:1, 2x), DCM (4x), ether (2x) and dried on vacuum gave solid supported triazolyl ligand (**8**).

Preparation of solid supported trizolyl ligand-Cu complex (9): To a solution of tetrakis methylcyano phosphorous hexaflouro copper (I) complex (0.08 g, 0.23 mmol) in DMF was added ligand **8** (0.5 g, 0.11 mmol) and kept on shaker at room temperature for overnight. Reaction was filtered and the resin was washed with DCM: methanol (9:1, 2x), DCM (4x), ether (2x) and further dried on vacuum to get Cu-complex (**9**). Loading of the Cu on resin was analyzed by ICP/OES, which gave 222 μmol/g, (97%).

Example 4

Chelated copper mediated click reaction of nucleoside with an azide 11

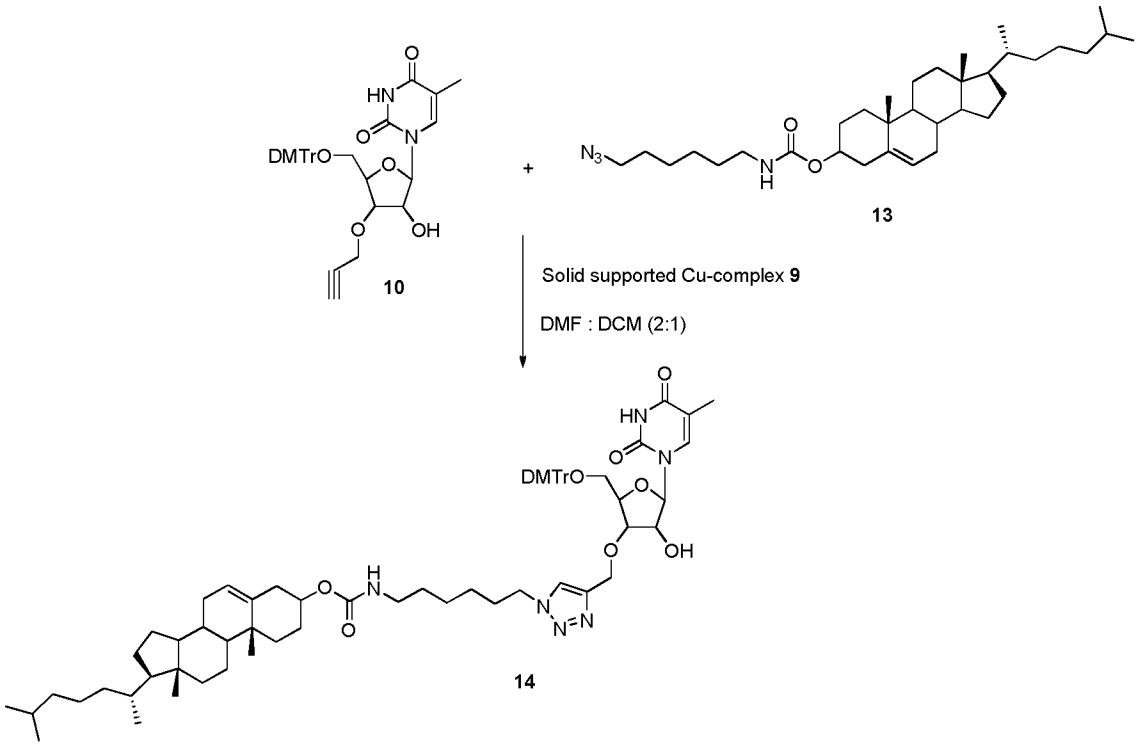


Preparation of Compound 12: To a mixture of alkyne **10** (0.05 g, 0.08 mmol, 1.0 eq) and azide **11** (0.03 g, 0.08 mmol, 1.0 eq) in DMF: DCM (2:1) was added Cu-complex **9** (0.05 g, 0.01 mmol, 0.12 eq) and the mixture was continued on shaker for overnight. After completion of the reaction was filtered, washed with DCM (2x), the solvent mixture was poured onto ice, extracted with DCM, separated the organic and washed with excess amount of water. Combined organics were dried on $MgSO_4$, evaporated and the mixture is purified by column chromatography using dichloromethane: methanol (5%) as gradients to get pure trizole compound (**12**).

Calc. mass for $C_{56}H_{79}N_5O_8$: 949.5; found 948.5 (M^{-1}).

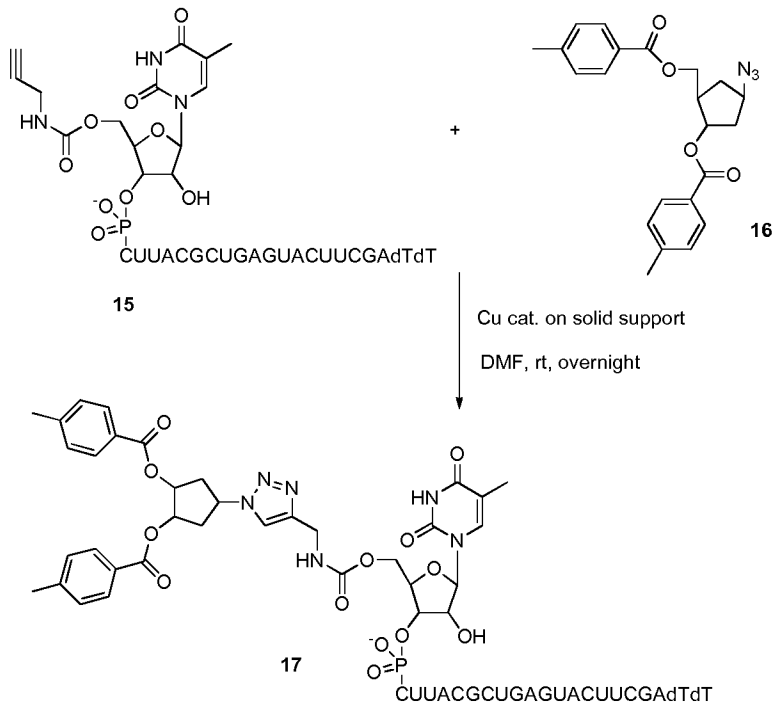
Recyclable ligand 9: Using ligand **9** in the repeated click reaction cycles gave quantitative yield of the product.

Example 5. Chelated copper mediated click reaction of nucleoside with an azide 13



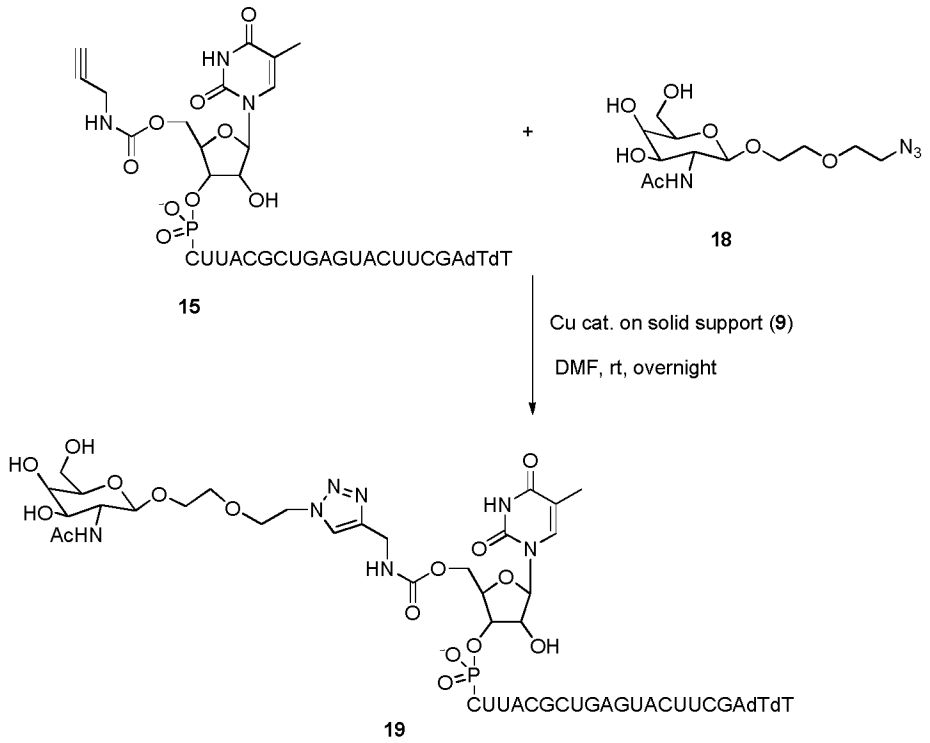
Preparation of Compound 14: Compound **14** has been prepared by using similar experimental conditions used for **12**, using alkyne **10** (0.05 g, 0.08 mmol, 1.0 eq), azide **13** (0.04 g, 0.08 mmol, 1.0 eq) and Cu-complex **9** (0.05 g, 0.01 mmol, 0.12 eq) in DMF: DCM (2:1) get pure trizole compound (**14**). Calc. mass for $C_{68}H_{92}N_6O_{10}$: 1152.6; found 1152.5.

Example 6. Chelated copper mediated click reaction of Oligonucleotide 15 with azide 16



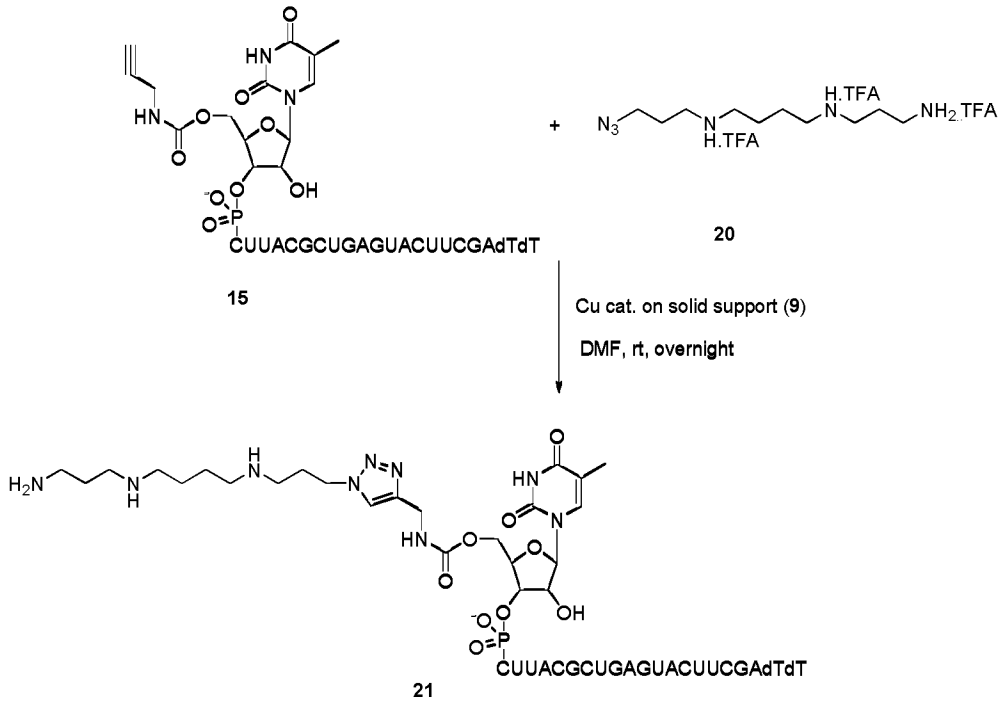
Preparation of Compound 17: Compound **17** has been prepared by using similar experimental conditions used for **12**, using oligo alkyne **15** (35.6 μmol in stock solution, 1.0 eq), azide **16** (71.0 μmol, 2.0 eq) and Cu-complex **9** (9.8 μmol, 0.2 eq) in DMF gave complete conversion of the product **17** in overnight at room temperature. Calc. mass for Compound **17** is 7402, observed 7402.

Example 7: Chelated copper mediated click reaction of Oligonucleotide 15 with azide 18



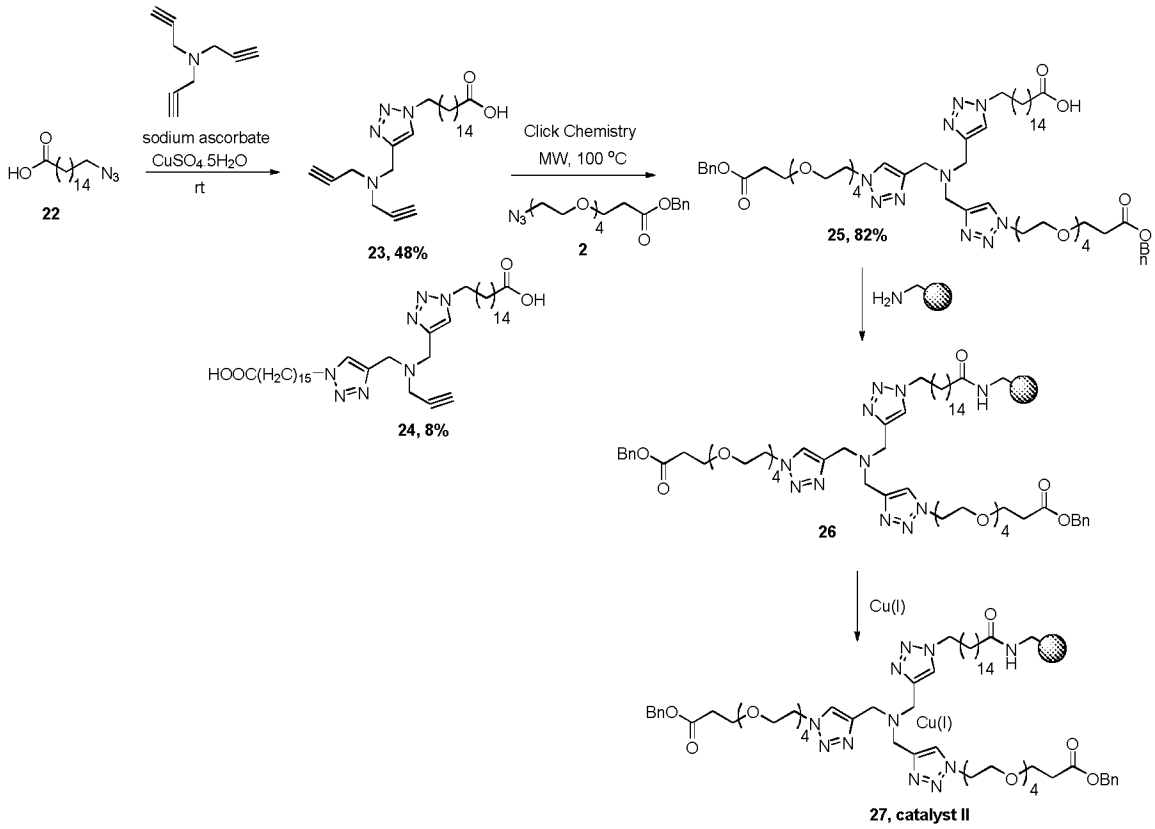
Preparation of Compound 19: Compound **19** has been prepared by using similar experimental conditions used for **12**, using oligo alkyne **15** (35.6 μmol in stock solution, 1.0 eq), azide **18** (71.0 μmol, 2.0 eq) and Cu-complex **9** (9.8 μmol, 0.2 eq) in DMF gave conversion of the product **19** in overnight at room temperature. Calc. mass for Compound **19** is 7341, observed 7342.

Example 8: Chelated copper mediated click reaction of Oligonucleotide 15 with azide 20



Preparation of Compound 21: Compound 21 has been prepared by using similar experimental conditions used for **12**, using oligo alkyne **15** (35.6 μmol in stock solution, 1.0 eq), azide **20** (71.0 μmol, 2.0 eq) and Cu-complex **9** (17.8 μmol, 0.5 eq) in DMF gave complete conversion of the product **21** in overnight at room temperature. Calc. mass for Compound **21** is 7235, observed 7235.

Example 9. Preparation of immobilized Cu Catalyst II:



Preparation of Compound 23: To the mixture of tris-tripropargylamine (1.3 g, 10.1 mmol, 3.0 eq) and azide **22** (1.0 g, 3.36 mmol, 1.0 eq) in tert-butanol : H_2O (1:1) at room temperature was added sodium ascorbate (0.2 g, 1.0 mmol, 0.3 eq) followed by $CuSO_4.5H_2O$ (0.02 g, 0.1 mmol, 0.03 eq) and continued the reaction for overnight. After Completion of the reaction, evaporated the organic solvent, dissolved in the dichloromethane and washed with water. Combined organics were dried on $MgSO_4$, concentrated and purified by column chromatography using DCM : MeOH (5%) as gradients to get pure mono triazolyl acid **23** (0. 7 g, 48%) and bis triazolyl compound **24** (0.2 g, 8%).

Preparation of Compound 25: To a solution of PEG-azide **2** (0.66 g, 1.75 mmol, 2.5 eq) and mono triazolyl acid **23** (0.3 g, 0.7 mmol, 1.0 eq) in t-butanol:water (1:1) were added sodium ascorbate (0.041 g, 0.21 mmol, 0.3 eq) followed by $CuSO_4.5H_2O$ (0.005g, 0.021 mmol, 0.03 eq) and the reaction was continued under microwave conditions at 100

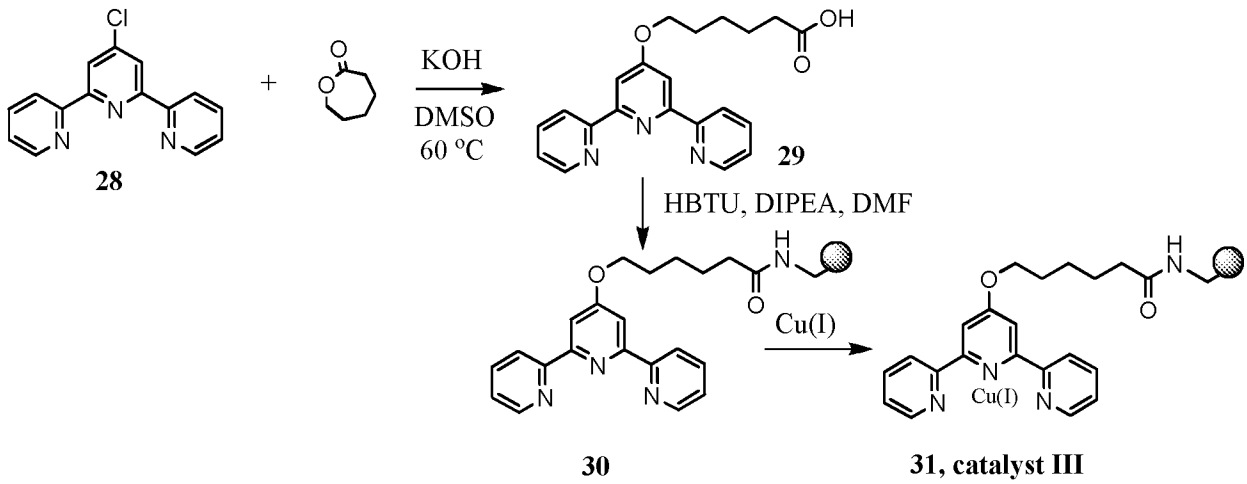
°C, TLC and LC Mass shows complete consumption of the starting materials after 1h. Reaction mixture was diluted with the dichloromethane, separated the organic layer and washed with water. Combined organics were dried on $MgSO_4$, concentrated, purified on column chromatography using dichloromethane and methanol (5%) as gradients to get pure tris-triazolyl ligand **25** as a white solid in 82% (0.7 g) yields.

Preparation of Compound 26: Ligand **25** was loaded on amino methyl polystyrene resin as similar conditions mentioned above using compound **25** (0.65 g, 0.55 mmol, 1.2 eq), HBTU (0.26 g, 0.69 mmol, 1.5 eq), DIPEA (0.2 mL, 1.3 mmol, 3.0 eq) and aminomethyl polystyrene resin (230 μmmol/g, 2.0 g, 0.46 mmol, 1.0), after complete dry, gave solid supported ligand **26** in 2.1 g yield.

Preparation of Cu Complex 27: Repeated by exact procedure mentioned above using tetrakis methylcyano phosphorous hexaflouro copper (I) complex (0.08 g, 0.23 mmol, 2.0 eq), ligand **26** (0.5 g, 0.11 mmol, 1.0 eq) in DMF, after completion of the reaction was filtered and the resin was washed with DCM: methanol (9:1, 2x), DCM (4x), ether (2x) and further dried on vacuum to get Cu-complex (**27**). Loading of the Cu on resin is analyzed by elemental analysis.

Example 10. Preparation of immobilized Cu Catalyst III:

Synthesis of Terpyridine ligand:



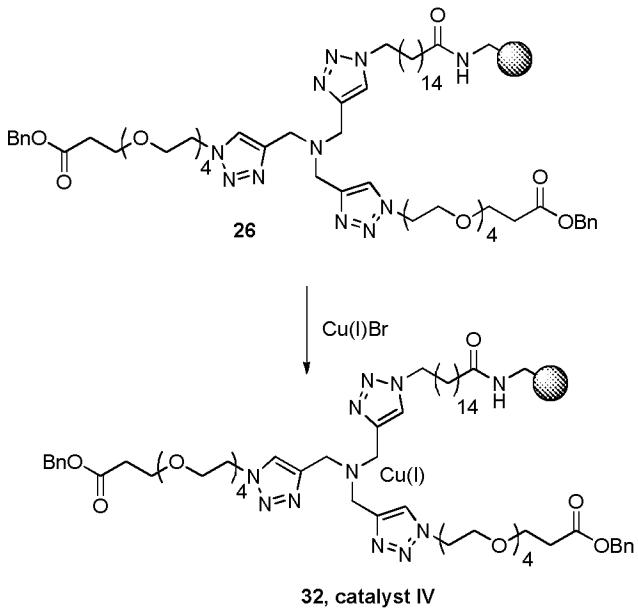
Preparation of Compound 29: ε-caprolactone was added dropwise to a stirred suspension of powdered KOH (1.0 g, 18.65 mmol, 5.0 eq) in DMSO at 60 oC. After 30

minutes, 4'-chloro-2,2',6',2"-terpyridine (**28**, 1.0 g, 3.73 mmol, 1.0 eq) was added as solid. The reaction mixture was stirred at 60 oC for 48h and then poured into water. Concentrated HCl wa added dropwise to the solution until precipitation of white solid formed (pH ~6.0). Precipitation was filtered and the product was dried on vacuum, which gave pure white solid **29** in 93% (1.26 g) yields.

Preparation of Compound 30: Repeated exactly as earlier using compound **29** (0.12 g, 0.34 mmol, 1.5 eq), HBTU (0.13 g, 0.34 mmol, 1.5 eq), DIPEA (0.12 mL, 0.7 mmol, 3.0 eq) and aminomethyl polystyrene resin (230 μmmol/g, 1.0 g, 0.23 mmol, 1.0), after complete dry, gave solid supported ligand **30** in 0.9 g yield.

Preparation of Cu Complex 31: Repeated by exact procedure mentioned above using tetrakis methylcyano phosphorous hexaflouro copper (I) complex (0.08 g, 0.23 mmol, 2.0 eq), ligand **30** (0.5 g, 0.11 mmol, 1.0 eq) in DMF, after completion of the reaction was filtered and the resin was washed with DCM: methanol (9:1, 2x), DCM (4x), ether (2x) and further dried on vacuum to get Cu-complex (**31**). Loading of the Cu on resin is analyzed by elemental analysis.

Example 11. Preparation of immobilized Cu Catalyst IV:

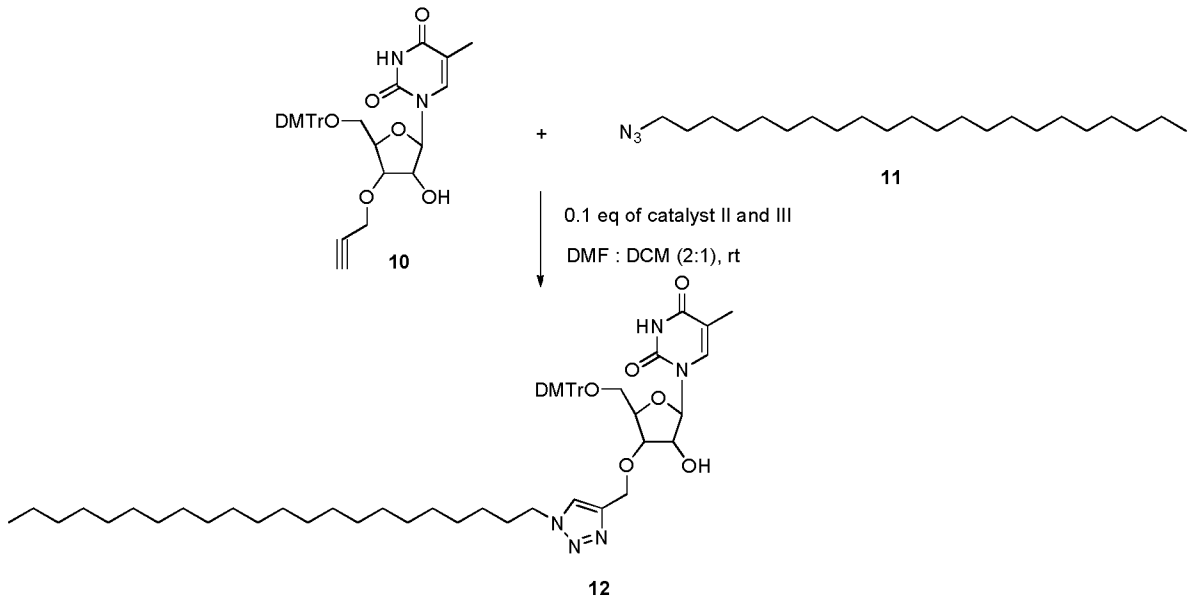


32, catalyst IV

Preparation of Cu Complex 32: Repeated by exact procedure mentioned above using Cu(I)Br (0.016 g, 0.11 mmol, 2.0 eq), ligand **26** (0.25 g, 0.057 mmol, 1.0 eq) in

DMF, after completion of the reaction was filtered and the resin was washed with DCM: methanol (9:1, 2x), DCM (4x), ether (2x) and further dried on vacuum to get Cu-complex (**32**). Loading of the Cu on resin is analyzed by elemental analysis.

Example 12: Click reaction of nucleoside 10 with azide 11 using Catalysts II, III:

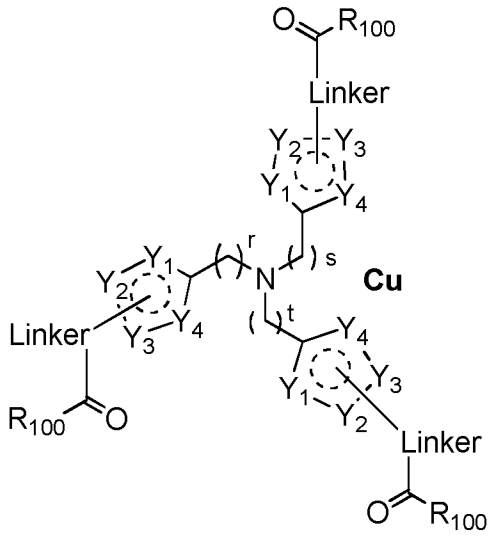


Preparation of Compound 12: Repeated exact procedure as earlier for the click reactions using catalyst **II** and **III** by using a mixture of alkyne **10** (0.03 g, 0.05 mmol, 1.0 eq) and azide **11** (0.017 g, 0.05 mmol, 1.0 eq) in DMF: DCM (2:1) and Cu-complex **II** and **III** (0.025 g, 0.005 mmol, 0.1 eq) to get the product **12** in complete conversion.

Calc. mass for $C_{56}H_{79}N_5O_8$: 949.5; found 948.5 (M^{-1}).

We claim:

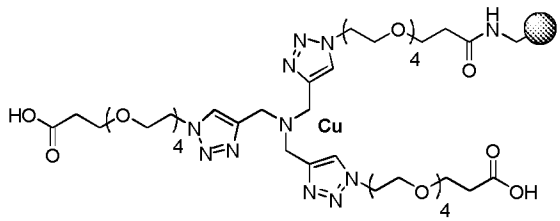
1. A chelated copper on solid support of formula A:



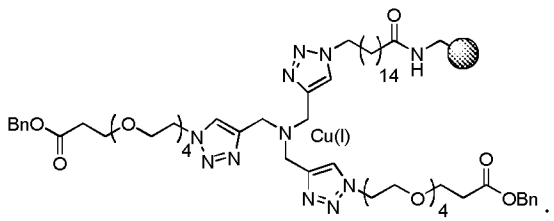
(A)

wherein Y_1-Y_4 are each independently CR^P, CR^P_2, N, NR^N, O, and S; r, s and t are each indenpendently 1-6; R_{100} is independently for each occurrence OH, OR^P, or solid support; R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl; R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; and each linker can be the same or different; provided that at least one R_{100} is bound to a solid support.

2. The chelated copper solid support of claim 1, selected from group consisting of:

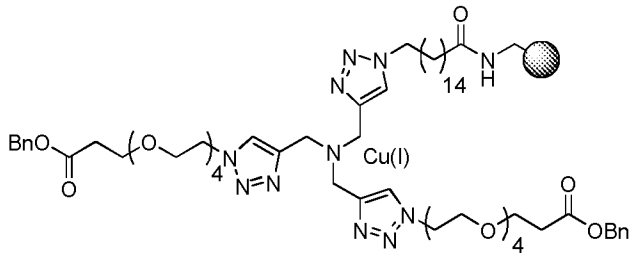


;



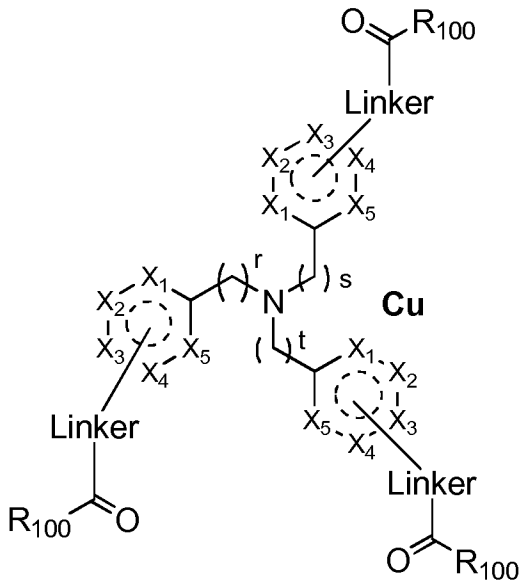
;

and



; where ● is a solid support.

3. A chelated copper on solid support of formula B:

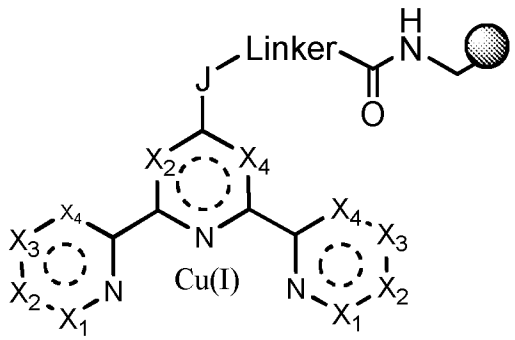


(B)

wherein X_1-X_5 are each independently CR^P, CR^P_2, N, NR^N, O, and S; r, s and t are each indenpendently 1-6; R_{100} is independently for each occurrence OH, OR^P, or solid support; R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl; R^N is independently

for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; and each linker can be the same or different; provided that at least one R_{100} is bound to a solid support.

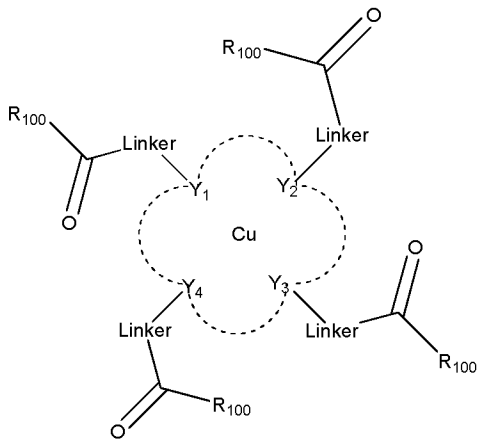
4. A chelated copper on solid support of formula C:



(C)

wherein X_1-X_4 are each independently CR^P, CR^P_2, N, NR^N, O, and S; J is absent, O, S, NR^N, OC(O)NH, NHC(O)O, C(O)NH, NHC(O), NHSO, $NHSO_2$, $NHSO_2NH$, OC(O), C(O)O, OC(O)O, NHC(O)NH, NHC(S)NH, OC(S)NH, $OP(N(R^P)_2)O$, or $OP(N(R^P)_2)$; R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl; R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; and  is a solid support.

5. A chelated copper on solid support of formula D:



(D)

wherein ---------- is a bond or absent; Y_1-Y_4 are each independently N, NR^N, O, and S; R_{100} is independently for each occurrence OH, OR^P, or solid support; R^P is independently for each occurrence occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl or optionally substituted heteroaryl; R^N is independently for each occurrence H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted cycloalkyl, optionally substituted aralkyl, optionally substituted heteroaryl or an amino protecting group; and each linker can be the same or different; provided that at least one R_{100} is bound to a solid support.

6. A process for preparing a ligand conjugated oligonucleotide comprising the step of contacting a nucleoside or oligonucleotide containing an alkyne moiety with ligand containing an azide moiety in the presence of a solid supported copper catalyst.

7. The process of claim 6, wherein the ligand is selected from the group consisting of lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, peptide mimics, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids,

multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic and an aptamer.

8. The process of claim 6, wherein the solid support copper catalyst is the chelated copper on solid support of claims 1, 3, or 4.

9. A process for preparing a ligand-conjugated oligonucleotide comprising the step of contacting a nucleoside or oligonucleotide containing an azide moiety with ligand containing an alkyne moiety in the presence of a solid supported copper catalyst.

10. The process of claim 9, wherein the ligand is selected from the group consisting of lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, peptide mimics, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic and an aptamer.

11. The process of claim 9, wherein the solid support copper catalyst is the chelated copper on solid support of claims 1, 3, or 4.