(19) DANMARK

(10) **DK/EP 2970968 T3**

Patent- og Varemærkestyrelsen

(12) Oversættelse af europæisk patentskrift

(51) Int.Cl.: *C 12 N 15/11 (2006.01)* *C 07 H 21/04 (2006.01)* *C 12 N 15/113 (2010.01)*

(45) Oversættelsen bekendtgjort den: **2018-03-05**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2018-01-10**

(86) Europæisk ansøgning nr.: **14765516.1**

(86) Europæisk indleveringsdag: **2014-03-16**

(87) Den europæiske ansøgnings publiceringsdag: **2016-01-20**

(86) International ansøgning nr.: **US2014030100**

(87) Internationalt publikationsnr.: **WO2014145356**

(30) Prioritet: **2013-03-15 US 201361791704 P**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Miragen Therapeutics, Inc., 6200 Lookout Road, Boulder, Colorado 80301, USA**

(72) Opfinder: **VAGLE, Kurt, c/o MIRAGEN THERAPEUTICS, 6200 Lookout Road, Boulder, Colorado80301, USA**

(74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**

(54) Benævnelse: **FORBUNDNE BICYKLISKE NUKLEOSIDER**

(56) Fremdragne publikationer:
WO-A1-99/60855
WO-A1-2011/085102
WO-A1-2011/115818
WO-A1-2011/156202
WO-A1-2013/036868
SINGH S K ET AL: "SYNTHESIS OF NOVEL BICYCLO not 2.2.1 3/4 RIBONUCLEOSIDES: 2'-AMINO- AND 2'-THIO-LNA MONOMERIC NUCLEOSIDES", THE JOURNAL OF ORGANIC CHEMISTRY, AMERICAN CHEMICAL SOCIETY, US, 1 January 1998 (1998-01-01), XP002901132, ISSN: 0022-3263, DOI: 10.1021/JO9806658

Description

FIELD OF THE INVENTION

[0001] The present invention relates to modified nucleosides, nucleotides, and oligonucleotides having advantages in synthesis, potency, efficiency of delivery, target specificity, stability, and/or toxicity when administered to a patient.

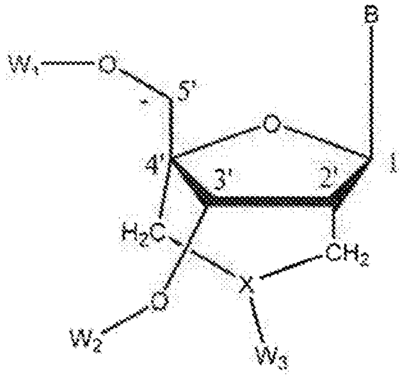
BACKGROUND

[0002] Oligonucleotide chemistry patterns or motifs for antisense oligonucleotide inhibitors have the potential to improve the delivery, stability, potency, specificity, and/or toxicity profile of the inhibitors, and such are needed for effectively targeting RNA function in a therapeutic context.

SUMMARY OF THE INVENTION

[0003] The present invention relates to modified nucleosides, nucleotides, and oligonucleotides comprising at least one 2'-C-Bridged Bicyclic Nucleotide (CBBN), and pharmaceutical compositions comprising the modified oligonucleotides. The invention further provides methods of use and synthesis for these oligonucleotides, as well as synthetic intermediates. In various embodiments, the oligonucleotides are antisense inhibitors that provide advantages in potency, efficiency of delivery, target specificity, stability, and/or toxicity.

[0004] In one aspect, the present invention provides 2'-C-Bridged Bicyclic Nucleosides or Nucleotides (CBBN) having the structure of formula I:



Formula I

wherein X is N ; W_1 and W_2 are independently H, an alcohol protecting group, phosphate ester, phosphorothioate ester, di- or tri-phosphate, or phosphoramidite; W_3 independently is null, H, O, an amine protecting group, phosphoramidite, phosphoramidate ester, phosphordiamidate ester, methyl, alkyl, cycloalkyl, carboxamide, a sugar, a fatty acid, or other conjugated molecules described herein, $-C_{1-4}(O)R$, or -COOR, wherein R is aryl, linear or cyclic alkyl or alkenyl, sugar, fatty acid, or other molecular conjugate such as a drug conjugate; B is a nucleobase. In some embodiments, the nucleobase is a pyrimidine base. In other embodiments, the nucleobase is a purine base.

[0005] In another aspect, the present invention provides oligonucleotides comprising at least one 2'-C-Bridged Bicyclic Nucleotide. The number of 2'-C-Bridged Bicyclic Nucleotides within the oligonucleotide may vary. For example, the number of 2'-C-Bridged Bicyclic Nucleotides may be at least about 10% of the nucleotides, at least about 25% of the nucleotides, at least about 50% of the nucleotides, or at least about 75% of the nucleotides. The length of the oligonucleotides may also vary. For example, the oligonucleotides of the present invention may be from about 5 to about 50 nucleotides in length or from about 10 to about 25 nucleotides in length. In other embodiments, the oligonucleotides may be less than about 10 nucleotides in length (e.g., from about 5 to about 10 nucleotides) or less than about 8 nucleotides in length (e.g., from about 5 to about 8 nucleotides).

[0006] The oligonucleotides may further include at least one nucleotide with a 2' modification selected from 2'-deoxy, 2'-O-methyl, 2'-fluoro, and a 2' to 4' bridge structure. In certain embodiments, the oligonucleotides further comprise backbone modifications such as phosphorothioate linkages and/or phosphorodiamidate linkages. In yet other embodiments, the oligonucleotides include at least one purine and/or pyrimidine base modifications. For example, the base modification may be at the C-5 position of a pyrimidine base and/or the C-8 position of a purine base. In some embodiments, the oligonucleotides include one or more morpholino nucleotides.

[0007] In various embodiments, the oligonucleotide comprises a nucleotide sequence that is at least substantially identical or complementary to a nucleotide sequence of a human microRNA. For example, the oligonucleotide may comprise a nucleotide sequence that is substantially complementary to a human miR-15a, miR-15b, miR-29, miR-92, miR-143, miR-145, miR-195, miR-206, miR-208a, miR-208b, miR-378, miR-451 and/or miR-499 sequence. In yet further embodiments, the oligonucleotide comprises a nucleotide sequence that is completely identical or complementary to a nucleotide sequence of a human microRNA. In various embodiments, the sequence of the oligonucleotide may be designed so as to mimic a miRNA or target a miRNA by antisense inhibition.

[0008] In another aspect, the present invention provides a pharmaceutical composition comprising an effective amount of the oligonucleotide described herein, or a pharmaceutically-acceptable salt thereof, and a pharmaceutically-acceptable carrier or diluent. The pharmaceutically-acceptable carrier may include a colloidal dispersion system, macromolecular complex, nanocapsule, microsphere, bead, oil-in-water emulsion, micelle, mixed micelle, or liposome. In an embodiment, the pharmaceutical composition is an aqueous formulation.

[0009] In yet another aspect, the present invention provides a method of reducing or inhibiting an RNA by antisense action, including reducing or inhibiting an mRNA target or microRNA target in a cell. The method comprises contacting a cell with the oligonucleotide disclosed herein. In various embodiments, the cell may be a mammalian cell. In one embodiment, the cell may be, for example, a heart cell. In another embodiment, the cell may be contacted with the oligonucleotide *in vivo* or ex *vivo*.

[0010] In a further aspect, the present invention provides a method of preventing or treating a condition in a subject associated with or mediated by a microRNA, comprising administering to the subject a pharmaceutical composition comprising an oligonucleotide targeting a miRNA as described herein. In various embodiments, the pharmaceutical composition is administered by parenteral administration or by direct injection into target tissue. In other embodiments, the pharmaceutical composition is administered by oral, transdermal, sustained release, controlled release, delayed release, suppository, catheter, or sublingual administration.

[0011] In still another aspect, the present invention provides a method of synthesizing a 2'-C-Bridged Bicyclic Nucleoside, a 2'-C-Bridged Bicyclic Nucleotide or a corresponding phosphoramidite, or a 2'-C-Bridged Bicyclic Nucleotide-containing oligonucleotide. The method comprises using, for example, ribose as a starting material, converting to a methyl 4,4-bismesyloxymethyl-2-hydroxymethylfuranose derivative, followed by glycosylation of the derivative. The 2-hydroxymethyl group of the glycosylated material is then converted to a protected amine that is then cyclized on the alpha face of the nucleoside with the corresponding 4-mesyloxymethyl group to give a fully protected aza-bicyclic nucleoside that is readily converted to a nucleoside phosphoramidite via standard protecting group chemistry. Alternative methods for synthesis are described in a U.S. provisional patent application by Kurt Vagle, entitled "Synthesis of Bicyclic Nucleosides," filed March 16, 2014. Other aspects and embodiments of the invention will be apparent from the following detailed description and examples.

DESCRIPTION OF THE DRAWINGS

[0012]

Figure 1 illustrates the synthesis of an amine 2'-C-Bridged Bicyclic Nucleoside.

Figure 2 illustrates 2'-C-Bridged Bicyclic Nucleosides linked by a phosphorodiamidate linkage.

Figure 3A illustrates an exemplary dimethoxytrityl (DMTr)-protected amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite. Figure 3B illustrates an internal phosphoramidite derivative of a DMTr-protected amine 2'-C-Bridged Bicyclic Nucleoside. Figure 3C illustrates an exemplary DMTr- and trifluoroacetate-protected amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite. Figure 3D illustrates an exemplary DMTr-protected fatty acid conjugated amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite. Figure 3E illustrates an exemplary DMTr-protected sugar conjugated amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite.

Figure 4A illustrates the synthesis of an exemplary dimethoxytrityl (DMTr)-protected amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite. Figure 4B illustrates the synthesis of an internal phosphoramidite derivative of a DMTr-protected amine 2'-C-Bridged Bicyclic Nucleoside. Figure 4C illustrates the synthesis of an exemplary DMTr-protected fatty acid conjugated amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite. Figure 4D illustrates the synthesis of an exemplary DMTr-protected sugar conjugated amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite.

Figure 5A provides a comparison chart of the affinity increases (ΔT_{m}, $_{C}$/modification) for locked nucleoside (LNA), its aminoLNA counterpart, as well as 2'-O,4'-C-Ethylene-Bridged Nucleoside (oxoENA) and its aminoENA counterpart. Figure 5B provides a comparison chart of the affinity increases (ΔT_{m}, $_{C}$/modification) for amine 2'-C-Bridged

Bicyclic Nucleoside (aminoCBBN) with its oxoCBBN counterpart. As shown, amine 2'-C-Bridged Bicyclic Nucleoside imparts much more affinity per modification than its oxoCBBN counterpart. Additionally, single and multiple aminoCBBN modifications within an oligonucleotide impart affinities equal to or greater than those of LNA nucleosides.

Figure 6 depicts the efficacy of various miR-208a inhibitors on miR-208a expression as measured in a dual-luciferase reporter assay. The activities of compounds M-10591, M-10101, M-11919, and M-11920 are measured. Compound M-10591 is a non-targeting control. Compound M-10101, a mixed 9 LNA/7 DNA phosphorothioate oligonucleotide, is an optimized miR208a inhibitor. The M10101 compound is described in U.S. Patent No. 8,642,751.

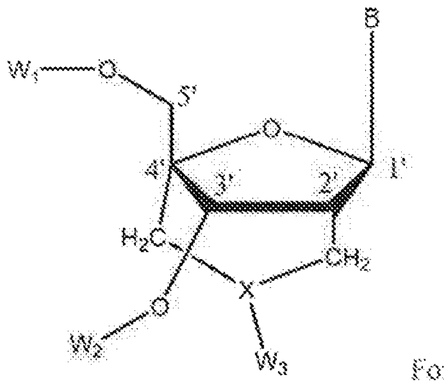
Compounds M-10919 and M-11920 are mixed LNA/DNA/aminoCBBN phosphorothioate oligonucleotides where LNA thymidines of the parent compound (M-10101) are replaced with either 1 or 2 aminoCBBN residues, respectively. As shown, compound M-11920, in which multiple LNA residues are replaced with aminoCBBN residues, retains all activity of the optimized M-10101 compound.

DETAILED DESCRIPTION OF THE INVENTION

[0013] The present invention relates to 2'-C-Bridged Bicyclic Nucleosides and Nucleotides, and oligonucleotides comprising at least one 2'-C-Bridged Bicyclic Nucleotide. The invention further provides pharmaceutical compositions comprising the modified oligonucleotides, as well as methods of use and synthesis for these oligonucleotides.

[0014] The oligonucleotides of the present invention in various embodiments can provide advantages in synthesis, potency, efficiency of delivery, target specificity, stability, and/or toxicity. In an exemplary embodiment, the oligonucleotides of the invention provide novel conjugation strategies allowing for advantages in targeting cells or tissues by attaching a ligand and/or a drug compound, to the bridging moiety such as an amine. In another embodiment, the oligonucleotides of the invention provide therapeutic advantages in potency, stability, and/or toxicity.

[0015] In one aspect, the present invention provides 2'-C-Bridged Bicyclic Nucleosides or Nucleotides, or oligonucleotides having the structure of Formula 1:



Formula I

wherein X is N. In certain aspects described herein but not claimed, X is S.

[0016] In various embodiments, W_1 and W_2 are independently H, an alcohol protecting group, phosphate ester, phosphorothioate ester, di- or tri-phosphate, or phosphoramidite. W_3 independently is null, H, O, an amine protecting group, phosphoramidite, phosphoramidate ester, phosphordiamidate ester, methyl, alkyl, cycloalkyl, carboxamide, a sugar, a fatty acid, or other conjugated molecules described herein, $-C_{1-4}(O)R$, or -COOR, wherein R is aryl, linear or cyclic alkyl or alkenyl, sugar, fatty acid, or other molecular conjugate such as a drug conjugate.

[0017] In various embodiments, the alcohol protecting group is selected from 4,4'-dimethoxytrityl, acetyl, silyl, or acid labile ether. In an embodiment, W_1 and W_2 each is an alcohol protecting group independently selected from 4,4'-dimethoxytrityl, acetyl, silyl, or acid labile ether. In various embodiments, the amine protecting group is carbobenzyloxy (Cbz), p-methoxybenzyl carbonyl (Moz or MeOZ), tert-butyloxycarbonyl (BOC), 9-fluorenylmethyloxycarbonyl (FMOC), acetyl (Ac), benzoyl (Bz), benzyl (Bn), trifluoroacetyl (tfa). In an embodiment, W_3 is an amine protecting group selected from carboxybenzyl, tert-butoxycarbonyl, or trifluoroacetamidyl.

[0018] In various aspects, 2'-C-Bridged Bicyclic Nucleoside is a 2'-deoxy-2'-C, 4'-C-Bridged Bicyclic Nucleoside (2'-CBBN).

[0019] In various aspects, oxo-2'-C-Bridged Bicyclic Nucleoside is a 2'-deoxy-2'-C, 4'-C-Bridged Bicyclic Nucleoside, wherein 2'C and 4'C are connected through a oxygen resulting in a three atom linkage (-C-O-C-) (oxoCBBN).

[0020] In various aspects, amino-2'-C-Bridged Bicyclic Nucleoside or aza-2'-Bridged Bicyclic Nucleoside is a 2'-deoxy-

2'-C, 4'-C-Bridged Bicyclic Nucleoside, wherein 2'C and 4'C are connected through a nitrogen resulting in a three atom linkage (-C-N-C-) (aminoCBBN).

[0021] In various aspects, thio-2'-C-Bridged Bicyclic Nucleoside is a 2'-deoxy-2'-C, 4'-C-Bridged Bicyclic Nucleoside, wherein 2'C and 4'C are connected through a sulfur resulting in a three atom linkage (-C-S-C-) (thioCBBN).

[0022] In various aspects, amino-2'-C-Bridged Bicyclic Nucleotide and thio-2'-C-Bridged Bicyclic Nucleotide are phosphoesters of the amino-2'-C-Bridged Bicyclic Nucleosides and thio-2'-C-Bridged Bicyclic Nucleosides, respectively.

[0023] In various aspects, locked nucleoside is a 2'-oxo-4'-C-Bridged Bicyclic Nucleoside (LNA) that has a 2 atom linkage between the 2' and 4' position of the nucleoside's ribose ring. The core sugar forms a 2.5-dioxabicyclo[2.2.1] heptane structure.

[0024] In various aspects, ENA and oxoENA is a 2'-oxo-4'-C-Bridged Bicyclic Nucleoside that has a 3 atom linkage between the 2' and 4' position of the nucleoside's ribose ring. The core sugar forms a 2.6-dioxabicyclo[3.2.1]octane structure.

[0025] In various aspects, aminoENA and azaENA is a 2'-aza-4'-C-Bridged Bicyclic Nucleoside that has a 3 atom linkage between the 2' and 4' position of the nucleoside's ribose ring. The core sugar forms 6-oxa-2-azabicyclo[3.2.1] octane structure.

[0026] In various embodiments, B is a nucleobase. The nucleobase or base can be a purine or a pyrimidine base, which may be modified. In one embodiment, the nucleobase is a purine base. In another embodiment, the nucleobase is a pyrimidine base. In various embodiments, the nucleobase can be selected from natural nucleosidic bases such as adenine, guanine, uracil, thymine, and cytosine, or derivatives and or substitutes thereof. In addition, the present invention also contemplates the use of non-naturally occurring nucleobases. In certain embodiments, the non-naturally occurring nucleobase can be a base in which any of the ring atoms of the nucleobases is replaced by another atom. For example, CH may be replaced by N and vice versa. Such modifications can occur at more than one position. Another example of a non-naturally occurring base is a base in which the 2- and 4-substituents of a naturally occurring base are reversed. Additional purine and/or pyrimidine base modifications are described in WO 2012/061810.

[0027] In some embodiments, the base modification is an amino carbonyl, such as a carboxamino, carbamoyl, or carbamide group. The modification in various embodiments is at the C-5 position of one or more pyrimidine bases, and/or at the C-8 position of one or more purine bases. Exemplary nucleobases include, but are not limited to, 9-N-adenine, 9-N-guanine, thymidine, cytidine, uridine, 5-methyl-cytosine, inosine, 5-substituted uridine, 5-substituted cytosine, 2-aminoadenosine or 5-methylcytosine.

[0028] In some embodiments, the 2-C-Bridged Bicyclic Nucleotides may be positioned as locked nucleotides as described in WO 2012/083005 and U.S. Patent Publication No. 2013/0345288. For example, the number and position of the 2'-C-Bridged Bicyclic Nucleotides may be such that the oligonucleotide reduces or inhibits miR-15a, miR-15b, miR-208a, miR-208b, and/or miR-499 activity at high potency. In certain embodiments, the oligonucleotide does not contain a stretch of nucleotides with more than four, or more than three, or more than two contiguous 2'-C-Bridged Bicyclic Nucleotides. In certain embodiments, the oligonucleotide does not contain a stretch of nucleotides with more than two contiguous non-2'-C-Bridged Bicyclic Nucleotides. For example, the oligonucleotide may have just one occurrence of contiguous non-2'-C-Bridged Bicyclic Nucleotides. In exemplary embodiments, the oligonucleotide has exactly 9 2'-C-Bridged Bicyclic Nucleotides and 7 non-2'-C-Bridged Bicyclic Nucleotides. For example, the pattern of 2'-C-Bridged Bicyclic Nucleotides may be such that at least positions 1, 6, 10, 13, and 15 are 2'-C-Bridged Bicyclic Nucleotides. In certain embodiments, at least positions 1, 5, 10, and 16 are 2'-C-Bridged Bicyclic Nucleotides. In certain embodiments, positions 1, 5, 6, 8, 10, 11, 13, 15, and 16 are 2'-C-Bridged Bicyclic Nucleotides, and the remaining positions are non-2'-C-Bridged Bicyclic Nucleotides. In other embodiments, positions 1, 3, 4, 5, 6, 8, 10, 13, 15, and 16 are 2'-C-Bridged Bicyclic Nucleotides, with the remaining positions being non-2'-C-Bridged Bicyclic Nucleotides. In still other embodiments, positions 1, 4, 5, 7, 9, 10, 12, 14, and 16 are 2'-C-Bridged Bicyclic Nucleotides, with remaining positions being non-2'-C-Bridged Bicyclic Nucleotides. The non-2'-C-Bridged Bicyclic Nucleotides may be non-locked nucleotides.

[0029] In some embodiments, the oligonucleotide is from about 5 to 50 nucleotides in length, from about 8 to 30 nucleotides in length, or from about 10 to 25 nucleotides in length, or from 12 to 16 nucleotides in length. In certain embodiments, the oligonucleotide is from about 5 to about 10 nucleotides in length, or about 5 to about 8 nucleotides in length. In certain embodiments, the oligonucleotide is about 8 nucleotides in length, about 9 nucleotides in length, about 10 nucleotides in length, about 11 nucleotides in length, about 12 nucleotides in length, about 13 nucleotides in length, about 14 nucleotides in length, about 15 nucleotides in length, or about 16 nucleotides in length. In certain embodiments, the oligonucleotide is about 16 nucleotides or less in length, about 12 nucleotides or less in length, about 10 nucleotides or less in length, or about 8 nucleotides or less in length. In some embodiments, the oligonucleotides of the present invention comprise at least 1, at least 3, at least 5, or at least 7 2'-C-Bridged Bicyclic Nucleotides. In some embodiments, the oligonucleotides of the present invention include only 1, 2, or 3 2'-C-Bridged Bicyclic Nucleotides. In certain embodiments, at least about 5%, at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, or 100% of the nucleotides are 2'-C-Bridged Bicyclic Nucleotides. In certain embodiments, the oligonucleotide is not fully comprised of 2'-C-Bridged Bicyclic Nucleotides. For example, embodiments described in this paragraph may be amino 2'-C-Bridged Bicyclic Nucleotides.

[0030] The oligonucleotides of the present invention may be DNA- or RNA-based, and/or may employ one or more nucleic acid modifications, for example, such as a modified oligonucleotide backbone or one or more modified nucleoside units. The oligonucleotide or derivative may have one or more single stranded and/or one or more double stranded regions. The oligonucleotide may be an antisense oligonucleotide, short interfering RNA (siRNA), double stranded RNA (dsRNA), single stranded RNA (ssRNA), microRNA (miRNA), short hairpin RNA (shRNA), or ribozyme.

[0031] In certain embodiments, the oligonucleotides of the present invention comprise at least one nucleotide having purine and/or pyrimidine base modifications as described in WO 2012/061810. In some embodiments, the base modification is generally an amino carbonyl, such as a carboxamino, carbamoyl, or carbamide group. The modification in various embodiments is at the C-5 position of one or more pyrimidine bases, and/or at the C-8 position of one or more purine bases.

[0032] In some embodiments, the oligonucleotide further comprises at least one nucleotide with a 2' modification. As used herein, the term "2' modification" includes any 2' group other than OH. In some embodiments the 2' modification may be independently selected from O-alkyl (which may be substituted), halo, deoxy (H), and a 2' to 4' methoxy bridged nucleotide. For example, the 2' modifications may each be independently selected from O-methyl and fluoro. In exemplary embodiments, purine nucleotides each have a 2' OMe and pyrimidine nucleotides each have a 2' F. In certain embodiments, from one to about five 2' positions, or from about one to about three 2' positions are left unmodified (e.g., as 2' hydroxyls).

[0033] The 2' modifications in accordance with the invention may be selected from small hydrocarbon substituents. The hydrocarbon substituents include alkyl, alkenyl, alkynyl, and alkoxyalkyl, where the alkyl (including the alkyl portion of alkoxy), alkenyl and alkynyl may be substituted or unsubstituted. The alkyl, alkenyl, and alkynyl may be C1 to C10 alkyl, alkenyl or alkynyl, such as C1, C2, or C3. The hydrocarbon substituents may include one or two or three non-carbon atoms, which may be independently selected from N, O, and/or S. The 2' modifications may further include the alkyl, alkenyl, and alkynyl as O-alkyl, O-alkenyl, and O-alkynyl.

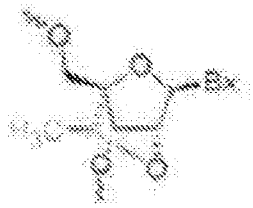
[0034] Exemplary 2' modifications in accordance with the invention include 2'-O-alkyl (C1-3 alkyl, such as 2'OMe or 2'OEt), 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) substitutions.

[0035] In certain embodiments, the oligonucleotide contains at least one 2'-halo modification (e.g., in place of a 2' hydroxyl), such as 2'-fluoro, 2'-chloro, 2'-bromo, and 2'-iodo. In some embodiments, the 2' halo modification is fluoro. The oligonucleotide may contain from one to about five 2'-halo modifications (e.g., fluoro), or from one to about three 2'-halo modifications (e.g., fluoro). In some embodiments, the oligonucleotide contains all 2'-fluoro nucleotides at non-2'-C-Bridged Bicyclic Nucleotides positions. In certain embodiments, the 2'-fluoro groups are independently di-, tri-, or un-methylated.

[0036] The oligonucleotide may have one or more 2'-deoxy modifications (e.g., H for 2' hydroxyl), and in some embodiments, contains from about one to about ten 2'-deoxy modifications.

[0037] The oligonucleotides of the invention may further include at least one locked nucleotide (LNA), as described, for example, in U.S. Patent No. 6,268,490, U.S. Patent No. 6,316,198, U.S. Patent No. 6,403,566, U.S. Patent No. 6,770,748, U.S. Patent No. 6,998,484, U.S. Patent No. 6,670,461, and U.S. Patent No. 7,034,133, all of which are hereby incorporated by reference in their entireties. In one embodiment, the oligonucleotide contains one or more LNAs having the structure shown by structure A below. Alternatively or in addition, the oligonucleotide contains one or more LNAs having the structure shown by structure B below.

A

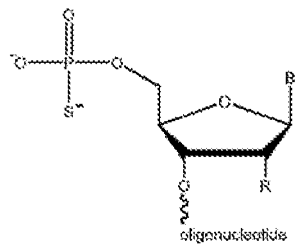


B

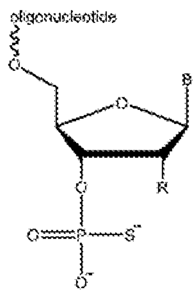
[0038] Other suitable locked nucleotides that can be incorporated in the oligonucleotides of the invention include those described in U.S. Patent No. 6,403,566 and U.S. Patent No.6,833,361,

[0039] In certain embodiments, the oligonucleotide further comprises at least one terminal modification or "cap". The cap may be a 5' and/or a 3'-cap structure. The terms "cap" or "end-cap" include chemical modifications at either terminus of the oligonucleotide (with respect to terminal ribonucleotides), and including modifications at the linkage between the last two nucleotides on the 5' end and the last two nucleotides on the 3' end. The cap structure as described herein may increase resistance of the oligonucleotide to exonucleases without compromising molecular interactions with the RNA target or cellular machinery. Such modifications may be selected on the basis of their increased potency *in vitro* or *in vivo*. The cap can be present at the 5'-terminus (5'-cap) or at the 3'-terminus (3'-cap) or can be present on both ends. In certain embodiments, the 5'- and/or 3'-cap is independently selected from phosphorothioate monophosphate, abasic residue (moiety), phosphorothioate linkage, 4'-thio nucleotide, carbocyclic nucleotide, phosphorodithioate linkage, inverted nucleotide or inverted abasic moiety (2'-3' or 3'-3'), phosphorodithioate monophosphate, and methylphosphonate moiety. The phosphorothioate or phosphorodithioate linkage(s), when part of a cap structure, are generally positioned between the two terminal nucleotides on the 5' end and the two terminal nucleotides on the 3' end.

[0040] In certain embodiments, the oligonucleotides have at least one terminal phosphorothioate monophosphate. The phosphorothioate monophosphate may be at the 5' and/or 3' end of the oligonucleotide. A phosphorothioate monophosphate is defined by the following structures, where B is base, and R is a 2' modification as described above:

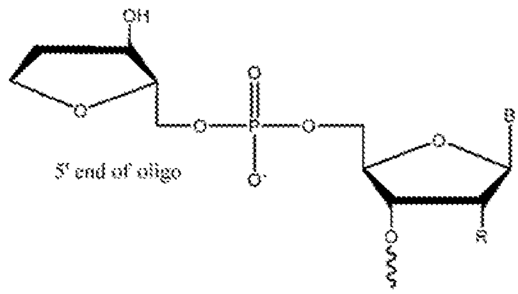


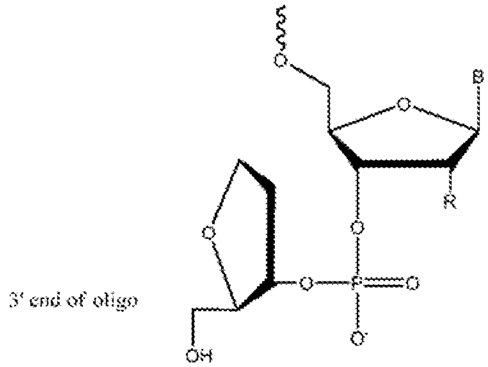
5' phosphorothioate monophosphate



3' phosphorothioate monophosphate

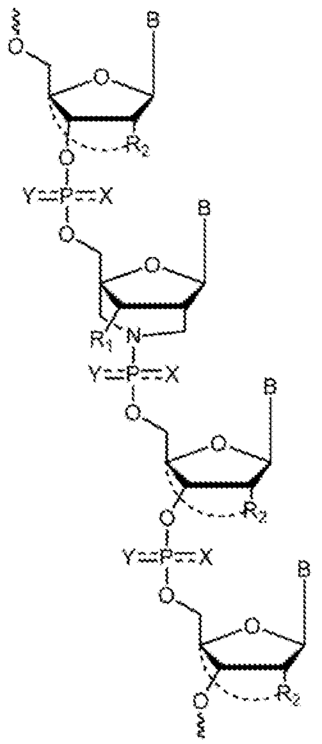
[0041] Phosphorothioate linkages may be present in some embodiments, such as between the last two nucleotides on the 5' and the 3' end (*e.g.*, as part of a cap structure), or as alternating with phosphodiester bonds. In these or other embodiments, the oligonucleotide may contain at least one terminal abasic residue at either or both the 5' and 3' ends. An abasic moiety does not contain a commonly recognized purine or pyrimidine nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine. Thus, such abasic moieties lack a nucleotide base or have other non-nucleotide base chemical groups at the 1' position. For example, the abasic nucleotide may be a reverse abasic nucleotide, *e.g.*, where a reverse abasic phosphoramidite is coupled via a 5' amidite (instead of 3' amidite) resulting in a 5'-5' phosphate bond. The structure of a reverse abasic nucleoside for the 5' and the 3' end of a polynucleotide is shown below.



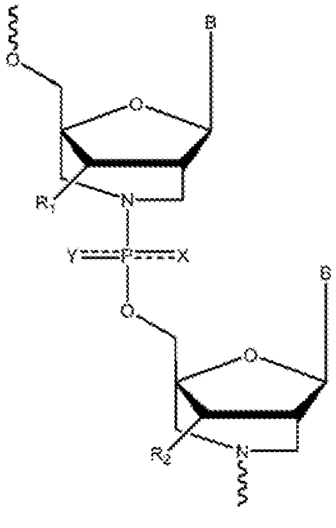


[0042] The oligonucleotide may contain one or more phosphorothioate linkages. Phosphorothioate linkages have been used to render oligonucleotides more resistant to nuclease cleavage. For example, the polynucleotide may be partially phosphorothioate-linked, for example, phosphorothioate linkages may alternate with phosphodiester linkages. In certain embodiments, however, the oligonucleotide is fully phosphorothioate-linked. In other embodiments, the oligonucleotide has from one to five or one to three phosphate linkages. In further embodiments, a 2'-C-Bridged Bicyclic Nucleotides are phosphorothioate linked.

[0043] In yet other embodiments, the oligonucleotides may include one or more modified phosphate linkages. Exemplary modified phosphate linkages are depicted below as Formulas II and III:



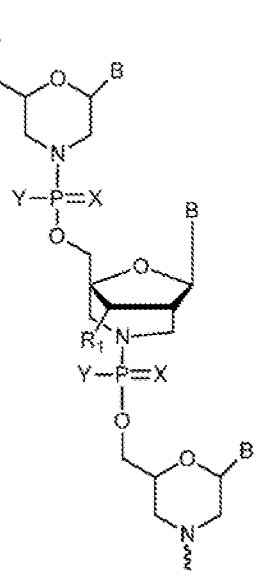
Formula II



Formula III

[0044] As shown, R_1 and R2 are independently selected from H, alkyl, alkenyl, oxo, aryl, benzyl, halogen, -OH, -NH2, alkoxy, an alcohol protecting group, or an amine protecting group, and with respect to formula II, a bicyclic linkage such as LNA, or 2'-C-Bridged Bicyclic Nucleoside. X and Y are independently selected from H, O, S, $-CO_2H$, alkyl, alkenyl, aryl, benzyl carboxylate, $-N(alkyl)_2$, $-N(alkenyl)_2$, $-N(aryl)_2$, $-N(benzyl)_2$, $-NH_2$, - BH_2, or borate ester. B may be an independently selected nucleobase such as a purine or pyrimidine base, which may be modified. As shown, the polynucleotide may be partially phosphorodiamidate-linked (as shown in Figure 2). In certain embodiments, however, the oligonucleotide is fully phosphorodiamidate linked. In other embodiments, the oligonucleotide has from one to five or one to three phosphate linkages. In further embodiments, 2'-C-Bridged Bicyclic Nucleotides are all linked by modified phosphate linkages (as shown in Formulas II and III).

[0045] In yet a further embodiment, the oligonucleotide may include one or more morpholino nucleotides. The morphlino nucleotides may be linked to 2'-C-Bridged Bicyclic Nucleosides as exemplified below in Formula IV:



Formula IV

[0046] As shown in formula IV, R_1 is independently selected from H, alkyl, alkenyl, oxo, aryl, benzyl, halogen, OH, -NH2, alkoxy, an alcohol protecting group, or an amine protecting group. X and Y are independently selected from H, O, S, -CO2H, alkyl, alkenyl, aryl, benzyl carboxylate, $-N(alkyl)_2$, $-N(alkenyl)_2$, $-N(aryl)_2$, $-N(benzyl)_2$, $-NH_2$, $-BH_2$, or Borate ester. B may be an independently selected nucleobase such as a purine or pyrimidine base, which may be modified.

[0047] The oligonucleotides disclosed herein may have a nucleotide sequence designed to inhibit an RNA molecule, including an mRNA or miRNA. In some embodiments, the oligonucleotide has a base sequence to mimic or target a mature miRNA, such as a mature miRNA listed in Table 1 below. The oligonucleotides may in these or other embodiments, be designed to target the pre- or pri-miRNA forms. In some embodiments, the oligonucleotides are substantially complementary to a nucleotide sequence of a human miRNA sequence. In further embodiments, the oligonucleotides are substantially identical to a nucleotide sequence of a human miRNA sequence. Exemplary oligonucleotides are at least partially complementary (*i.e.*, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 95%, or 100% complementary) to a target miRNA sequence, such as a mature miRNA sequence listed in Table 1 below. Such antisense and sense sequences may be incorporated into shRNAs or other RNA structures containing stem and loop portions, for example. Such sequences are useful for, among other things, mimicking or targeting miRNA function for treatment or ameliorating cardiac hypertrophy, myocardial infarction, heart failure (*e.g.*, congestive heart failure), vascular damage, and/or pathologic cardiac fibrosis, among others. Exemplary miRNA therapeutic utilities are disclosed in the US and PCT patent references listed in Table 2 below.

[0048] The mature and pre-processed forms of miRNAs are disclosed in the patent references listed below.

Table 1

miRNA	miRNA Sequence	Reference
1	UGGAAUGUAAAGAAGUAUGUAU (SEQ ID No. 1)	WO 2009/012468
100	AACCCGUAGAUCCGAACUUGUG (SEQ ID No. 2)	WO 2009/012468
10b	UACCCUGUAGAACCGAAUUUGUG (SEQ ID No. 3)	WO 2009/012468
125b	UCCCUGAGACCCUAACUUGUGA (SEQ ID No. 4)	WO 2009/012468
128	UCACAGUGAACCGGUCUCUUU (SEQ ID No. 5)	WO 2007/070483
133a	UUUGGUCCCCUUCAACCAGCUG (SEQ ID No. 6)	WO 2009/012468
133b	UUUGGUCCCCUUCAACCAGCUA (SEQ ID No. 7)	WO 2009/012468

(continued)

miRNA	miRNA Sequence	Reference
139	UCUACAGUGCACGUGUCUCCAG (SEQ ID No. 8)	WO 2009/012468
143	UGAGAUGAAGCACUGUAGCUC (SEQ ID No. 9)	WO 2007/070483
145	GUCCAGUUUUCCCAGGAAUCCCU (SEQ ID No. 10)	WO 20071070483
150	UCUCCCAACCCUUGUACCAGUG (SEQ ID No. 11)	WO 2009/012468
15a	UAGCAGCACAUAAUGGUUUGUG (SEQ ID No. 12)	WO 2009/062169
15b	UAGCAGCACAUCAUGGUUUACA (SEQ ID No. 13)	WO 2009/062169
16	UAGCAGCACGUAAAUAUUGGCG (SEQ ID No. 14)	WO 2009/062169
181b	AACAUUCAUUGCUGUCGGUGGGU (SEQ ID No. 15)	WO 2009/012468
195	UAGCAGCACAGAAAUAUUGGC (SEQ ID No. 16)	WO 2009/012468
197	UUCACCACCUUCUCCACCCAGC (SEQ ID No. 17)	WO 2009/012468
199a	CCCAGUGUUCAGACUACCUGUUC (SEQ ID No. 18)	WO 2009/012468
199b	miR-199b-5p CCCAGUGUUUAGACUAUCUGUUC (SEQ ID No. 19) miR-199b-3p ACAGUAGUCUGCACAUUGGUUA (SEQ ID No. 20)	WO 2010/135570
206	UGGAAUGUAAGGAAGUGUGUGG (SEQ ID No. 21)	WO 2007/070483
208a	AUAAGACGAGCAAAAAGCUUGU (SEQ ID No. 22)	WO 2008/016924
208b	AUAAGACGAACAAAAGGUUUGU (SEQ ID No. 23)	WO 2009/018492
20a	UAAAGUGCUUAUAGUGCAGGUAG (SEQ ID No. 24)	US 60/950,565
21	UAGCUUAUCAGACUGAUGUUGA (SEQ ID No. 25)	WO 2009/058818
214	ACAGCAGGCACAGACAGGCAGU (SEQ ID No. 26)	US 61/047,005
22	AAGCUGCCAGUUGAAGAACUGU (SEQ ID No. 27)	WO 2009/012468
221	AGCUACAUUGUCUGCUGGGUUUC (SEQ ID No. 28)	WO 2009/012468
222	AGCUACAUCUGGCUACUGGGU (SEQ ID No. 29)	WO 2009/012468
224	CAAGUCACUAGUGGUUCCGUU (SEQ ID No. 30)	WO 2009/012468
23a	AUCACAUUGCCAGGGAUUUCC (SEQ ID No. 31)	WO 2009/012468
26a	UUCAAGUAAUCCAGGAUAGGCU (SEQ ID No. 32)	WO 2007/070483
26b	UUCAAGUAAUUCAGGAUAGGU (SEQ ID No. 33)	WO 2009/012468
28	AAGGAGCUCACAGUCUAUUGAG (SEQ ID No. 34)	WO 2009/012468
29a	UAGCACCAUCUGAAAUCGGUUA (SEQ ID No. 35)	WO 2009/018493
29b	UAGCACCAUUUGAAAUCAGUGUU (SEQ ID No. 36)	WO 2009/018493
29c	UAGCACCAUUUGAAAUCGGUUA (SEQ ID No. 37)	WO 2009/018493
30a	UGUAAACAUCCUCGACUGGAAG (SEQ ID No. 38)	WO 2010/120969
30b	UGUAAACAUCCUACACUCAGCU (SEQ ID No. 39)	WO 2010/120969
30c	UGUAAACAUCCUACACUCUCAGC (SEQ ID No. 40)	WO 2009/012468
30d	UGUAAACAUCCCCGACUGGAAG (SEQ ID No. 41)	WO 2010/120969
30e	UGUAAACAUCCUUGACUGGAAG (SEQ ID No. 42)	WO 2010/120969
342-3p	UCUCACACAGAAAUCGCACCCGU (SEQ ID No. 43)	WO 2009/012468

(continued)

miRNA	miRNA Sequence	Reference
382	GAAGUUGUUCGUGGUGGAUUCG (SEQ ID No. 44)	WO 2009/012468
422a	ACUGGACUUAGGGUCAGAAGGC (SEQ ID No. 45)	US 2009/0226375
378	ACUGGACUUGGAGUCAGAAGG (SEQ ID No. 46)	WO 2009/012468
424	CAGCAGCAAUUCAUGUUUUGAA (SEQ ID No. 47)	WO 2009/062169
483-3p	UCACUCCUCUCCUCCCGUCUU (SEQ ID No. 48)	WO 2009/012468
484	UCAGGCUCAGUCCCCUCCCGAU (SEQ ID No. 49)	WO 2009/012468
486-5p	UCCUGUACUGAGCUGCCCCGAG (SEQ ID No. 50)	WO 2009/012468
497	CAGCAGCACACUGUGGUUUGU (SEQ ID No. 51)	WO 2009/062169
499	UUAAGACUUGCAGUGAUGUUU (SEQ ID No. 52)	WO 2009/018492
542-5p	UCGGGGAUCAUCAUGUCACGAGA (SEQ ID No. 53)	WO 2009/012468
92a	UAUUGCACUUGUCCCGGCCUGU(SEQ ID No. 54)	WO 2009/012468
92b	UAUUGCACUCGUCCCGGCCUCC (SEQ ID No. 55)	WO 2009/012468
let-7a	UGAGGUAGUAGGUUGUAUAGUU (SEQ ID No. 56)	WO 2009/012468
let-7b	UGAGGUAGUAGGUUGUGUGGUU (SEQ ID No. 57)	WO 2009/012468
let-7c	UGAGGUAGUAGGUUGUAUGGUU (SEQ ID No. 58)	WO 2009/012468
let-7d	AGAGGUAGUAGGUUGCAUAGUU (SEQ ID No. 59)	WO 2009/012468
let-7e	UGAGGUAGGAGGUUGUAUAGUU (SEQ ID No. 60)	WO 2009/012468
let-7f	UGAGGUAGUAGAUUGUAUAGUU (SEQ ID No. 61)	WO 2009/012468
let-7g	UGAGGUAGUAGUUUGUACAGUU (SEQ ID No. 62)	WO 2009/012468
451	AAACCGUUACCAUUACUGAGUU (SEQ ID No. 63)	WO 2010/129950

Table 2

miRNA	Direction of Modulation	Indications	Reference
miR-208a/miR-208b/miR-499	Antagonist	Pathologic cardiac hypertrophy, myocardial infarction, heart failure	WO 2008/016924 (208a) WO 2009/018492 (208b/499)
miR-208a/miR-208b	Antagonist	Metabolic Disorders (obesity, hyperlipidemia, diabetes, metabolic syndrome, hypercholesterolemia; hepatic steatosis)	PCT/US2012/059349
miR-15/miR-16/miR-195	Antagonist	Pathologic cardiac hypertrophy, myocardial infarction, heart failure	WO 2009/062169
miR-29	Agonist	Tissue fibrosis (cardiac, pulmonary, hepatic, kidney)	WO 2009/018493
miR-29	Antagonist	profibrotic agents to convert soft plaques to fibrotic tissue	WO 2009/018493
miR-126	Agonist	promotes angiogenesis, vascular integrity, and vascular repair	WO 2010/019574
miR-126	Antagonist	pathologic vascularization	WO 2010/019574
miR-206	Agonist	Muscle injury	WO 2007/070483

(continued)

miRNA	Direction of Modulation	Indications	Reference
miR-206/miR-1	Agonist	Denervating neuropathic states (ALS, spinal cord injury, myasthenia gravis)	WO 2009/117418
miR-143	Agonist	Restenosis/neointima formation	WO 2009/105759
miR-1/miR-133	Agonist/ Anta gonist	Muscle injury (antagonist/agonist of each miRNA applied in combination at different times)	WO 2007/070483
miR-451	Antagonist	Polycythemia	WO 2012/148373
miR-451	Agonist	Anemia	WO 2012/148373
miR-378/miR-378*	Antagonist	Metabolic disorders (obesity, hyperlipidemia, diabetes, metabolic syndrome, hypercholesterolemia; hepatic steatosis); Pathologic cardiac hypertrophy, myocardial	WO 2011/153542
		infarction, heart failure	
miR-92	Antagonist	Promotes angiogenesis and vessel repair	US 2010/0324118 A1
miR-92	Agonist	Inhibits tumor angiogenesis	US 2010/0324118 A1
miR-34a	Antagonist	Myocardial infarction	US 2012/0238619 A1
miR-145	Antagonist	Pulmonary arterial hypertension	WO 2012/153135
miR-33	Antagonist	Statin-induced hepatotoxicity, cholestasis, increasing HDL cholesterol	US 20110281933 A1

[0049] In some embodiments, the oligonucleotides comprise a sequence that is substantially complementary to a nucleotide sequence of miR-15a or b, miR-29, miR-92, miR-143, miR-145, miR-195, miR-206, miR-208a, miR-208b, miR-378, miR-451 and/or miR-499. In exemplary embodiments, the oligonucleotides may comprise a sequence that is substantially complementary to a human miR-208a, miR-208b, miR-378, miR-451 and/or miR-499 sequence. In certain embodiments, the oligonucleotides may comprise a sequence that is substantially identical to a human miR-208a, miR-208b, miR-378, miR-451 and/or miR-499 sequence. As used herein, "substantially complementary" or "substantially identical" refers to a sequence that is at least about 70%, about 75%, about 80%, about 85%, about 90%, about 91 %, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary or identical to a target polynucleotide sequence.

[0050] The present invention further provides a method of synthesizing a 2'-C-Bridged Bicyclic Nucleoside, a 2'-C-Bridged Bicyclic Nucleotide or a corresponding phosphoramidite, or a 2'-C-Bridged Bicyclic Nucleotide-containing oligonucleotide. The method comprises using, for example, ribose as a starting material, converting to a methyl 4,4-bismesyloxymethyl-2-hydroxymethylfuranose derivative, followed by glycosylation of the derivative. The 2-hydroxymethyl group of the glycosylated material is then converted to a protected amine that is then cyclized on the alpha face of the nucleoside with the corresponding 4-mesyloxymethyl group to give a fully protected aza-bicyclic nucleoside that is readily converted to a nucleoside phosphoramidite via standard protecting group chemistry,. Exemplary synthetic schemes for the 2'-C-Bridged Bicyclic Nucleoside are shown herein. Alternative methods for synthesis are described in a U.S. provisional patent application by Kurt Vagle, entitled "Synthesis of Bicyclic Nucleosides," filed March 16, 2014.

[0051] The synthesis of oligonucleotides, including modified polynucleotides, by solid phase synthesis is well known and is reviewed by Caruthers et al., "New Chemical Methods for Synthesizing Polynucleotides," Nucleic Acids Symp. Ser., (7):215-23 (1980).

[0052] The synthesis of oligonucleotides will vary depending on the selected nucleotide monomer(s) utilized. In exemplary embodiments, the nucleotide monomers used for synthesis include, but are not limited to, dimethoxytrityl (DMTr)-protected amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite, an internal phosphoramidite derivative of a DMTr-protected amine 2'-C-Bridged Bicyclic Nucleoside, DMTr- and trifluoroacetate-protected amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite, DMTr-protected fatty acid conjugated amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite, and DMTr-protected sugar conjugated amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite (see Figures 3A - 3E, respectively). In certain embodiments, extended coupling time may be required for oligonucleotide synthesis utilizing dimethoxytrityl (DMTr)-protected amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite, DMTr- and trifluoroacetate-protected amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite, DMTr-protected fatty acid conjugated amine 2'-C-Bridged Bicyclic Nucleoside phosphoramidite, and DMTr-protected sugar conjugated amine 2-C-Bridged Bicyclic Nucleoside phosphoramidite. In certain embodiments, for oligonucleotide synthesis involving an internal phosphoramidite derivative of a DMTr-protected amine 2'-C-Bridged Bicyclic Nucleoside, the standard oligonucleotide synthesis cycle may be modified by replacing the normal capping reagent utilizing Ac_2O/base with a non-standard capping reagent. Alternatively, synthesis may be modified by treating the newly coupled oligonucleotide with an amine reactive conjugate or protecting group that is stable to the synthesis cycle (but if desired, can be removed later) immediately after the phosphoramidite coupling cycle, but before the standard capping step.

[0053] The oligonucleotide may be incorporated within a variety of macromolecular assemblies or compositions. Such complexes for delivery may include a variety of liposomes, nanoparticles, and micelles, formulated for delivery to a patient. The complexes may include one or more fusogenic or lipophilic molecules to initiate cellular membrane penetration. Such molecules are described, for example, in U.S. Patent No. 7,404,969 and U.S. Patent No. 7,202,227.

[0054] Alternatively, the oligonucleotide may further comprise a pendant lipophilic group to aid cellular delivery, such as those described in WO 2010/129672.

[0055] In another aspect, the present invention relates to a pharmaceutical composition which comprises an effective amount of the oligonucleotide of the present invention or a its pharmaceutically-acceptable salt thereof, and a pharmaceutically-acceptable carrier or diluent.

[0056] The composition or formulation may employ a plurality of therapeutic oligonucleotides, including at least one described herein. For example, the composition or formulation may employ at least about 2, about 3, about 4, or about 5 miRNA inhibitors described herein.

[0057] The oligonucleotides of the invention may be formulated as a variety of pharmaceutical compositions. Pharmaceutical compositions will be prepared in a form appropriate for the intended application. Generally, this entails preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals. Exemplary delivery/formulation systems include colloidal dispersion systems, macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. Commercially available fat emulsions that are suitable for delivering the nucleic acids of the invention to cardiac and skeletal muscle tissues include Intralipid®, Liposyn®, Liposyn® II, Liposyn® III, Nutrilipid, and other similar lipid emulsions. A preferred colloidal system for use as a delivery vehicle *in vivo* is a liposome (i.e., an artificial membrane vesicle). The preparation and use of such systems is well known in the art. Exemplary formulations are also disclosed in U.S. Patent No. 5,981,505; U.S. Patent No. 6,217,900; U.S. Patent No. 6,383,512; U.S. Patent No. 5,783,565; U.S. Patent No. 7,202,227; U.S. Patent No. 6,379,965; U.S. Patent No. 6,127,170; U.S. Patent No. 5,837,533; U.S. Patent No. 6,747,014; and WO 2003/093449.

[0058] The pharmaceutical compositions and formulations may employ appropriate salts and buffers to render delivery vehicles stable and allow for uptake by target cells. Aqueous compositions of the present invention comprise an effective amount of the delivery vehicle comprising the presently claimed oligonucleotide (*e.g.* liposomes or other complexes), dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically acceptable" or "pharmacologically acceptable" refers to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically acceptable carrier" may include one or more solvents, buffers, solutions, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like acceptable for use in formulating pharmaceuticals, such as pharmaceuticals suitable for administration to humans. The use of such media and agents for pharmaceutically active substances is well known in the art. Supplementary active ingredients also can be incorporated into the compositions.

[0059] Administration or delivery of the pharmaceutical compositions according to the present invention may be via any route so long as the target tissue is available via that route. For example, administration may be by intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection, or by direct injection into target tissue (e.g., cardiac tissue). The stability and/or potency of the oligonucleotides disclosed herein allows for convenient routes of administration, including subcutaneous, intradermal, and intramuscular. Pharmaceutical compositions comprising miRNA inhibitors may also be administered by catheter systems or systems that isolate coronary circulation for delivering therapeutic agents to the heart. Various catheter systems for delivering therapeutic agents to the heart and coronary vasculature are known in the art. Some non-limiting examples of catheter-based delivery methods or coronary isolation methods suitable for use in the present invention are disclosed in U.S. Patent No. 6,416,510; U.S. Patent No. 6,716,196; U.S. Patent No. 6,953,466, WO 2005/082440, WO 2006/089340, U.S. Patent Publication No. 2007/0203445, U.S. Patent Publication No. 2006/0148742, and U.S. Patent Publication No. 2007/0060907.

[0060] The compositions or formulations may also be administered parenterally or intraperitoneally. By way of illustration, solutions of the conjugates as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations generally contain a preservative to prevent the growth of microorganisms.

[0061] The pharmaceutical forms suitable for injectable use or catheter delivery include, for example, sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. Generally, these preparations are sterile and fluid to the extent that easy injectability exists. Preparations should be stable under the conditions of manufacture and storage and should be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Appropriate solvents or dispersion media may contain, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial an antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0062] Sterile injectable solutions may be prepared by incorporating the conjugates in an appropriate amount into a solvent along with any other ingredients (for example as enumerated above) as desired. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the desired other ingredients, *e.g.*, as enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation include vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient(s) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0063] Upon formulation, solutions are preferably administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations may easily be administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like. For parenteral administration in an aqueous solution, for example, the solution generally is suitably buffered and the liquid diluent first rendered isotonic for example with sufficient saline or glucose. Such aqueous solutions may be used, for example, for intravenous, intramuscular, subcutaneous and intraperitoneal administration. Preferably, sterile aqueous media are employed as is known to those of skill in the art, particularly in light of the present disclosure. By way of illustration, a single dose may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by the FDA Office of Biologics standards.

[0064] In another aspect, the present invention provides a method of reducing or inhibiting RNA expression or activity in a cell. In such embodiments, the method comprises contacting the cell with an oligonucleotide disclosed herein (or pharmaceutical composition thereof), where the oligonucleotide hybridizes (e.g., is at least substantially complementary) to an RNA transcript expressed by the cell. In some embodiments, the RNA is an mRNA or a miRNA.

[0065] In another aspect, the present invention provides a method of preventing or treating a condition in a subject associated with or mediated by RNA or expression thereof. In some embodiments, the RNA is a mRNA or a miRNA. The method of prevention or treatment according to the present invention involves administering to the subject a pharmaceutical composition which comprises an effective amount of the oligonucleotide or its pharmaceutically-acceptable composition thereof.

[0066] The invention provides a method for delivering the oligonucleotides of the present invention to a mammalian cell (e.g., as part of a composition or formulation described herein), and methods for treating, ameliorating, or preventing the progression of a condition in a mammalian patient. The oligonucleotide or pharmaceutical composition may be contacted *in vitro* or *in vivo* with a target cell (*e.g.*, a mammalian cell). The cell may be a heart cell.

[0067] The method generally comprises administering the oligonucleotide or composition comprising the same to a mammalian patient or population of target cells. The oligonucleotide, as already described, may be a miRNA inhibitor (*e.g.*, having a nucleotide sequence designed to inhibit expression or activity of a miRNA). For example, where the miRNA inhibiter is an inhibitor of a miR-208 family miRNA, the patient may have a condition associated with, mediated by, or resulting from, miR-208 family expression. Such conditions include, for example, cardiac hypertrophy, myocardial infarction, heart failure (e.g., congestive heart failure), vascular damage, restenosis, or pathologic cardiac fibrosis, cancer, or other miRNA associated disorder, including those disorders described in the patent publication listed in Table 2. Thus, the invention provides a use of the modified oligonucleotides and compositions of the invention for treating such conditions, and for the preparation of medicaments for such treatments.

[0068] In certain embodiments, the patient (e.g., human patient) has one or more risk factors including, for example, long standing uncontrolled hypertension, uncorrected valvular disease, chronic angina, recent myocardial infarction, congestive heart failure, congenital predisposition to heart disease and pathological hypertrophy. Alternatively or in addition, the patient may have been diagnosed as having a genetic predisposition to, for example, cardiac hypertrophy, or may have a familial history of, for example, cardiac hypertrophy.

[0069] In this aspect, the present invention may provide for an improved exercise tolerance, reduced hospitalization, better quality of life, decreased morbidity, and/or decreased mortality in a patient with heart failure or cardiac hypertrophy.

[0070] In certain embodiments, the activity of microRNA in cardiac tissue, or as determined in patient serum, is reduced or inhibited.

[0071] In various embodiments, the pharmaceutical composition is administered by parenteral administration or by direct injection into heart tissue. The parenteral administration may be intravenous, subcutaneous, or intramuscular. In some embodiments, the composition is administered by oral, transdermal, sustained release, controlled release, delayed release, suppository, catheter, or sublingual administration. In certain embodiments, the oligonucleotide is administered at a dose of about 50 mg/kg or less, a dose of about 25 mg/kg or less, a dose of about 10 mg/kg or less, or a dose of about 5 mg/kg or less. In these embodiments, the oligonucleotide or composition may be administered by intramuscular or subcutaneous injection, or intravenously.

[0072] In some embodiments, the methods further comprise scavenging or clearing the miRNA inhibitors following treatment. For example, an oligonucleotide having a nucleotide sequence that is complementary to the inhibitor may be administered after therapy to attenuate or stop the function of the inhibitor.

EXAMPLES

Example 1: Production of 2'-C-Bridged Bicyclic Nucleotides

[0073] This example describes an exemplary synthesis of key intermediates for the production of amine 2'-C-Bridged Bicyclic Nucleotides (see Figure 1).

Methyl-D-Ribose (2)

[0074] In three 500 mL Schott Bottles were D-ribose (1) (90 g, 599 mmol), Amberlyst 15 (H+) (90 g, 599 mmol), and Molecular Trap Pack (90 g, 599 mmol) divided equally (i.e. 30g each in each Schott bottle). Each bottle was filled with an equal amount of methanol (Volume: 1350 ml, i.e. 450 mL/bottle) to give a colorless solution. All bottles were placed on an orbital shaker @ 250 rpm/25°C for 17 hours. Reaction progress was monitored by TLC of the reaction mixture compared to co-spot with unprotected ribose in 15% MeOH/DCM as developing solvent. The sugars were visualized via Hannessian's Stain with charring.

[0075] The solutions were filtered through a glass sintered funnel. The catalyst and Molecular Trap Packs were washed with excess MeOH (~500 mL/ Bottle that contained 30g each of Amberlyst and Trap Packs). The methanol solution was made basic by addition of 15 mL of TEA (5 mL/reaction bottle). The mixtures were concentrated to dryness. The residue was co-evaporated with dichloromethane (3 x 200 mL) to azeotrope off residual MeOH. The residue was dried under high vacuum overnight to give 97.55g (99%) of methyl-D-ribose **(2)** which was used without further purification.

Methyl 5-O-(TBDPS)-α,β-D-ribofuranoside(3)

[0076] In a 1 L round-bottomed flask was methyl-**D**-ribose (**2**, 60.12 g, 366 mmol) and DIEA (128 ml, 732 mmol) dissolved in DMF (Volume: 400 ml) to give a colorless solution. The flask was flushed with argon and cooled to 0°C in an ice bath. TBDPS-Cl (99 ml, 385 mmol) was added dropwise over 10 minutes and the mixture was allowed to come to room temperature overnight.

[0077] The reaction mixture was poured into a solution of saturated $NaHCO_3$ (1 L). The aqueous phase was extracted with EtOAc (3 x 300mL). The organic phases were combined and washed with water (1 x 400 mL) and brine (1 x 400 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give a dark brown oil that was purified by dividing into 4 equal portions and purifying via silica chromatography running a standard 0-100% EtOAc/Hex gradient over 75 minutes at 100 mL/min followed by a 7 minute hold @ 100% EtOAc. Pure fractions were combined to give 121.59 g (82%) of methyl 5-O-(TBDPS)-α,β-D-ribofuranoside **(3)** as a colorless oil.

Methyl 5-O-(TBDPS)-2,3-O-bis(4-Chlorobenzyl)-α,β-D-ribofuranoside (4)

[0078] In a 2 L round-bottomed flask was weighed Methyl 5-O-(TBDPS)-α,β-D-ribofuranoside (3, 55.0 g, 137 mmol). The material was co-evaporated with toluene (2 x 100 mL) at 40°C and high vacuum. The flask was fitted with a reflux condenser and the starting material was dissolved under argon in Toluene (Volume: 500 ml). Sodium hydride (21.86 g, 547 mmol) was added in ~5 g portions to give a gray suspension. The mixture was heated to 60°C for 30 minutes and then cooled to room temperature with an ice bath. 1-chloro-4-(chloromethyl)benzene (66.0 g, 410 mmol) was added in ~15 g portions with vigorous stirring. The mixture was heated and stirred overnight at reflux.

[0079] The reaction mixture was cooled to 0°C and diluted with 500 mL of EtOAc. The mixture was quenched by slow addition of EtOH (50 mL) to minimize bubbling. The mixture was further diluted to 1.5 L with EtOAc and Washed with 10% Na_2CO_3 (2 x 500 mL) and sat NaCl (1 x 500 mL). The organic was dried over Na_2SO_4, filtered and concentrated. The crude product was purified via silica gel chromatography. Product was eluted with a 0-30% EtOAc/Hexanes gradient. Pure collected fractions were combined to give methyl 5-O-(TBDPS)-2,3-O-bis(4-chlorobenzyl)-α,β-D-ribofuranoside (**4**, 62.15 g, 70%) as an amber oil.

Methyl 5-O-(TBDPS)-3-O-(4-Chlorobenzyl)-α-D-ribofuranoside (5)

[0080] In a 1 L round-bottomed flask was methyl 5-O-(TBDPS)-2,3-O-bis(4-chlorobenzyl)-α,β-D-ribofuranoside (**4**, 65 g, 100 mmol) dissolved in 600 mL DCM to give a yellow solution. The mixture was cooled to 0°C under argon. Tin (IV) Chloride (150 ml, 150 mmol) was added slowly over 10 minutes while solution turns to a clear, dark brown solution. The reaction mixture was stored overnight at 4°C, under argon with stirring.

[0081] The reaction mixture was diluted with DCM (250 mL) and added to 500 mL of DI water in a 4L sep funnel. The mixture was shaken vigorously and allowed to separate. All organic and emulsion/precipitate was retained and washed with a second aliquot of 500 mL water. All organic and emulsion/precipitate was retained and washed with 500 mL of 10% Na_2CO_3 in water. The emulsion was reduced via addition of MeOH and mechanical agitation. All organic and emulsion/precipitate was retained and finally washed with 500 mL brine. Again, the emulsion was reduced via addition of MeOH and mechanical agitation. The organic phase was removed and dried via $MgSO_4$ suspension. The remaining emulsion and aqueous phase was extracted with additional DCM (2 x 100 mL) which was combined with the $MgSO_4$ suspension. The organic phase was filtered and concentrated to a brown oil. The crude product was purified via silica gel column chromatography with a 0-30% EtOAc/Hexanes gradient. Pure collected fractions were combined to give methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-α-D-ribofuranoside (**5**, 41.20g, 78%) as an amber oil.

Methyl 5-O-(TBDPS)-3-O-(4-Chlorobenzyl)-2-oxo-α-D-ribofuranoside (6)

[0082] In a 1 L round-bottomed flask was dissolved methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-α-D-ribofuranoside (**5**, 41.00 g, 78 mmol) and TEMPO (1.215 g, 7.78 mmol) in DCM (Volume: 250 ml) to give an orange solution. Iodobenzene diacetate (37.6 g, 117 mmol) was added and the mixture was allowed to stir overnight at room temperature.

[0083] Reaction mixture was diluted to 500 mL with DCM and washed with saturated sodium thiosulfate solution (2 x 300 mL), and brine (1 x 300 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated. The orange residue was dried under high vacuum at 50 °C for 3 hours. The crude methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-2-oxo-α-D-ribofuranoside (**6**, 40.50, "99%") as an amber oil was used as is for subsequent reaction.

Methyl 5-O-(TBDPS)-3-O-(4-Chlorobenzyl)-2-methylene-α-D-ribofuranoside (7)

[0084] In a 2000 mL round-bottomed flask was methyltriphenylphosphonium bromide (**6**, 26.6 g, 75 mmol) was suspended in ether (Ratio: 20.00, Volume: 1500 ml) to give a white suspension. The flask was flushed with argon and cooled to 0°C in an ice bath. Sodium t-pentoxide (7.39 g, 67 mmol) was dissolved in Benzene (Ratio: 1.000, Volume: 75ml) and added at once to the suspension. The flask was again flushed with argon and allowed to come to room temperature over 2 hours. The suspension was allowed to stir for an additional 4 hours. The suspension was then cooled to -72°C in an Acetone/dry ice bath. methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-2-oxo-α-D-ribofuranoside (19.56 g, 37.25 mmol) was dissolved in additional Ether (Ratio: 1.067,Volume: 40 ml). The carbohydrate solution was added via syringe and the reaction mixture was allowed to stir at 4°C for 17 hours.

[0085] TLC revealed that the reaction was complete (15% EtOAc/Hex). The reaction mixture was washed with sat NH_4Cl (2 x 500 mL) and brine (1 x 250 mL). The aqueous phase was back-extracted with Ether (150 mL). The organic phases were combined and dried with a brine wash (1 x 250 mL) and addition of Na_2SO_4. The organic phase was filtered and concentrated. Purification was done via silica gel column chromatography using a 0-20% EtOAc in Hexanes gradient. Pure fractions were combined and concentrated to dryness to give methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-2-deoxy-2-methylene-α-D-ribofuranoside (7, 14.79 g, 28.3 mmol, 76 % yield) as a colorless oil.

Methyl 5-O-(TBDPS)-3-O-(4-Chlorobenzyl)-2-deoxy-2-α-Hydroxymethyl-α-D-Ribofuranoside (8)

[0086] Under argon, 9-BBN (8.97 g, 73.5 mmol) was added to a solution of methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-2-deoxy-2-methylene-α-D-ribofuranoside (7, 28.50 g, 54.5 mmol) in THF (300 ml) at room temperature. After the reaction mixture was stirred at room temperature for 1.5 hours, TLC revealed that all starting material was consumed.

[0087] Sodium perborate tetrahydrate (33.9 g, 221 mmol) and water (80 mL) were added and the mixture was stirred at room temperature for an additional 2 hours. The organic layer was separated, and the aqueous was diluted to 400 mL then extracted with ethyl acetate (3 x 250 mL). The organic layers were combined and dried over $MgSO_4$. The solvent was removed, and the product was purified by silica gel chromatography eluting with ethyl acetate/hexanes gradient of 0-60%. The purified fractions were combined and concentrated to dryness to give methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-2-deoxy-2-α-hydroxymethyl-α-D-ribofuranoside (**8**, 26.39 g, 48.8 mmol, 90 % yield) as a colorless oil.

Methyl 3-O-(4-Chlorobenzyl)-2-deoxy-2-α-(4,4'-Dimethoxytrityloxymethyl)-α-D-Ribofuranoside (10)

[0088] In a 1 L round-bottomed flask was methyl 5-O-(TBDPS)-3-O-(4-chlorobenzyl)-2-deoxy-2-α-hydroxymethyl-α-D-ribofuranoside (**8**, 26.30 g, 48.6 mmol) in pyridine (200 ml) dissolved under Argon to give a colorless solution. DMTr-Cl (20.58 g, 60.8 mmol) was added, at once, to the stirring solution. The reaction mixture was allowed to stir overnight. The trytilation reaction was quenched by the addition of 50 mL of MeOH with stirring for 20 minutes followed by diluting the mixture to 750 mL with EtOAc. The Organic phase was washed with saturated $NaHCO_3$ solution (3 x 350 mL) and Brine (1 x 150 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to dryness.

[0089] The crude product (**9**) was dissolved in THF (Volume: 70 ml). 1.0 M TBAF in THF solution (72.9 ml, 72.9 mmol) was added to the mixture and it was allowed to stir at room temperature for 1.5 hours. Addition of the TBAF resulted in a dark, smoky colored solution. The mixture was concentrated to dryness and applied to a 330g ISCO silica column pretreated with 3% TEA in hexanes. The product was eluted with a 0-60% EtOAc in Hexanes gradient over 50 minutes @ 100 mL/min. The pure fractions were combined and concentrated to give methyl 3-O-(4-chlorobenzyl)-2-deoxy-2-α-(4,4'-dimethoxytrityloxymethyl)-α-D-ribofuranoside (**10**, 27.17 g, 44.9 mmol, 92 % yield) as a colorless oil.

Methyl 5-Oxo-3-O-(4-Chlorobenzyl)-2-deoxy-2-α-(4,4'-Dimethoxytrityloxymethyl)-α-D-Ribofuranoside (11)

[0090] In a 1 L round-bottomed flask was methyl 3-O-(4-chlorobenzyl)-2-deoxy-2-a-(4,4'-dimethoxytrityloxymethyl)-α-D-ribofuranoside (**10**, 27.15 g, 44.9 mmol) and DCC (27.8 g, 135 mmol) dissolved in DMSO (166 ml, 2333 mmol) to give a colorless solution. Pyridine (5.44 ml, 67.3 mmol) and TFA (1.728 ml, 22.43 mmol) were combined in 40 mL of DMSO and the resulting solution was added to the reaction mixture. The flask was covered and allowed to stir overnight at room temperature.

[0091] Water (25 mL) was added and the reaction was allowed to stir at room temperature for 3 hours. The reaction was diluted with 500 mL EtOAc and filtered. The precipitate was washed with an additional 200 mL of EtOAc. The combined organic was washed with Brine (5 x 400 mL), dried with Na_2SO_4, filtered and concentrated. The product was purified via silica gel column chromatography with a 0-100% EtOAc/Hex gradient. Pure fractions were combined and concentrated to give methyl 5-oxo-3-O-(4-chlorobenzyl)-2-deoxy-2-α-(4,4'-dimethoxytrityloxymethyl)-α-D-ribofuranoside (**11**, 25.22 g, 41.8 mmol, 93 % yield) as a white foam.

Methyl 4-C-Hydroxymethyl-3-O-(4-Chlorobenzyl)-2-deoxy-2-α-(4,4'-Dimethoxytrityloxymethyl)-α-D-Ribofuranoside (12)

[0092] In a 2 L round-bottomed flask was methyl 5-oxo-3-O-(4-chlorobenzyl)-2-deoxy-2-α-(4,4'-dimethoxytrityloxymethyl)-α-D-ribofuranoside (**11**, 25.20 g, 41.8 mmol) dissolved in Dioxane (1000 ml) to give a colorless solution. Formaldehyde (249 ml, 3343 mmol) was added with stirring. The reaction mixture was cooled to 0°C in an ice bath. The flask was fitted with a 750 mL pressure equalizing dropping funnel and 2.0 M sodium hydroxide (606 ml, 1212 mmol) was added over 30 minutes to give a cloudy white solution. The mixture was allowed to stir while coming to room temperature over 42 hours. The solution had turned clear. The solution was neutralized by addition of sodium phosphate, monobasic, monohydrate (86 g, 627 mmol). The solution was concentrated to about a third of its volume, diluted with 500 mL of water and extracted with DCM (3 x 300 mL). The organic layers were combined and washed with brine (1 x 300 mL) then dried over Na_2SO_4. The solvent was removed, and the product was purified by silica gel chromatography eluting with a MeOH/DCM gradient of 0-10%. The purified fractions were combined and concentrated to dryness to give methyl 4-C-hydroxymethyl-3-O-(4-chlorobenzyl)-2-deoxy-2-α-(4,4'-dimethoxytrityloxymethyl)-α-D-ribofuranoside (**12**, 22.50 g, 35.4 mmol, 85 % yield) as a colorless oil.

Methyl 5-O-Mesyl-4-C-(Mesyloxymethyl)-3-O-(4-Chlorobenzyl)-2-deoxy-2-α-(Hydroxymethyl)-α-D-Ribofuranoside (14)

[0093] In a 1 L round-bottomed flask was methyl 4-hydroxymethyl-3-O-(4-chlorobenzyl)-2-deoxy-2-α-(4,4'-dimethoxytrityloxymethyl)-α-D-ribofuranoside (**12**, 22.50 g, 35.4 mmol) dissolved in Pyridine (200 ml) under Ar to give a colorless solution. The mixture was cooled to 0°C in an ice bath. Mesyl-Cl (8.28 ml, 106 mmol) was added, dropwise over 10 minutes, to the stirring solution. The reaction mixture was stirred for 45 minutes at room temperature. The mesylation reaction was quenched by cooling the reaction to 0 °C and adding 15 mL of Water with stirring for 20 minutes. The mixture was diluted to 750 mL with EtOAc and washed with brine (3 x 400 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to dryness.

[0094] The crude product (13) was dissolved in 800 mL of AcOH. Water (200 mL) was added to the stirring solution. The solution was allowed to stir at room temperature for 2.5 hours then diluted with 500 mL of water. The mixture was concentrated to about 400 mL and diluted with an additional 250 mL of water. The solution was then concentrated to dryness under high vacuum. The residue was applied to a 220g ISCO silica column and the product was eluted with a 0-100% EtOAc/Hexanes gradient. The pure fractions were combined and concentrated to give methyl 5-O-mesyl-4-C-(mesyloxymethyl)-3-O-(4-chlorobenzyl)-2-deoxy-2-α-(Hydroxymethyl)-α-D-ribofuranoside (**14**, 10.01 g, 20.47 mmol, 57.8 % yield) as a colorless oil.

((2S,3R,4S)-2-acetoxy-4-((4-chlorobenzyl)oxy)-5,5-bis(((methylsulfonyl)oxy)methyl)tetrahydrofuran-3-yl)methyl acetate (16)

[0095] ((3S,4R,5S)-3-((4-chlorobenzyl)oxy)-4-(hydroxymethyl)-5-methoxytetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (3.41 g, 6.97 mmol) was weighed into a 100ml round-bottomed flask with a stir bar and septum sealed. The flask was cooled to 0°C and charged with pyridine (Volume: 25 ml) and acetic anhydride (1.316 ml, 13.95 mmol). The mixture was allowed to come to room temperature over 6 hours. The mixture was cooled to 0°C and MeOH (1 mL) was added and allowed to stir for 15 minutes. The mixture was concentrated to dryness and re-dissolved in EtOAc (100 mL). The organic phase was washed with aqueous 1% HCl (50 mL), saturated sodium bicarbonate (50 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated.

[0096] The resultant oil was re-dissolved with acetic acid (9.98 ml, 174 mmol) and acetic anhydride (2.63 ml, 27.9 mmol) in a 100 mL round-bottomed flask. H_2SO_4 (0.037 ml, 0.697 mmol) was added, the flask septum sealed and the mixture was allowed to stir overnight. The mixture was diluted with water (100 mL) and extracted with EtOAc (3 x 75 mL). The organic phases were combined and washed carefully with saturated sodium bicarbonate (2 x 100 mL) and brine (1 x 100 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give 3.15g of crude ((2S,3R,4S)-2-acetoxy-4-((4-chlorobenzyl)oxy)-5,5-bis(((methylsulfbnyl)oxy)methyl)tetrahydrofuran-3-yl)methyl acetate (3.15 g, 5.64 mmol, 81 % yield) as a pale yellow oil that was used without further purification.
ESI-MS: 617 (M + Acetate)$^-$

((3R,4S)-4-((4-chlorobenzyl)oxy)-2-(thymidin-yl)-5,5-bis(((methylsulfonyl)oxy)methyl)tetrahydrofuran-3-yl)methyl acetate (17)

[0097] N,O-Bis(trimethylsilyl)acetamide (4.07 ml, 16.64 mmol) was added to a mixture of ((3R,4S)-2-acetoxy-4-((4-chlorobenzyl)oxy)-5,5-bis(((methylsulfonyl)oxy)methyl)tetrahydrofuran-3-yl)methyl acetate (3.10 g, 5.55 mmol) and thymine (0.874 g, 6.93 mmol) in anhydrous acetonitrile (20 ml). The reaction mixture was refluxed for 1 hour to get a clear solution. The solution was cooled to 40°C and TMS-OTf (1.303 ml, 7.21 mmol) was added. The mixture was heated at 60°C for 4 hours. The solution was cooled to room temperature, diluted with CH_2Cl_2 (100 mL), and washed with saturated $NaHCO_3$ (2 x 100 mL) and brine (1 x 100mL). The organic layer was dried (Na_2SO_4), concentrated under reduced pressure, and the residue was purified by silica gel column chromatography on a standard Biotage Isolera gradient (0-10% v/v $MeOH/CH_2Cl_2$) to give ((3R,4S)-4-((4-chlorobenzyl)oxy)-2-(thymidin-yl)-5,5-bis(((methylsulfonyl)oxy)methyl)tetrahydrofuran-3-yl)methyl acetate (2.84 g, 4.54 mmol, 82 % yield) as a white solid material.
ESI-MS: 624 (M)$^-$

((3S,4R)-3-((4-chlorobenzyl)oxy)-4(hydroxymethyl)-5-(thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (19)

[0098] In a 100 mL round-bottomed flask fitted with a stir bar, ((3R,4S)-4-((4-chlorobenzyl)oxy)-2-(thymidin-yl)-5,5-bis(((methylsulfonyl)oxy)methyl)tetrahydrofuran-3-yl)methyl acetate (2.84 g, 4.54 mmol) was dissolved in Methanol (Volume: 20 ml). Sodium methoxide (0.123 g, 2.272 mmol) was added and the flask was covered and allowed to stir overnight at room temperature. TLC (100% EtOAc) revealed that the reaction was complete. The reaction mixture was evaporated to dryness *in vacuo*, and applied directly to a 3 g Biotage Samplet, which was fitted to a 25g Biotage SNAP column. The product was eluted with a 40-100% EtOAc/Hex gradient to give ((3S,4R)-3-((4-chlorobenzyl)oxy)-4-(hydroxymethyl)-5-(thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (2.32 g, 3.98 mmol, 88 % yield) as a white foam.
ESI-MS: 582 (M)$^-$

((3S,4R)-5-(thymidin-yl)-3-((4-chlorobenzyl)oxy)-4-(hydroxymethyl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (20)

[0099] To a mixture of ((3S,4R)-3-((4-chlorobenzyl)oxy)-4-(hydroxymethyl)-5-(thymidinyl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (1.0 g, 1.715 mmol) and pyridine (10 ml) was added TMS-C1 (0.219 ml, 1.715 mmol) at room temperature. After stirring for 1 hour, the reaction mixture was cooled to 0°C, and benzoyl chloride (0.199 ml, 1.715 mmol) was added dropwise by syringe. The ice-bath was then removed and the reaction mixture stirred at room temperature for 48 hours. The reaction was quenched by the addition of water (2mL); after stirring for 15 minutes at room temperature, the mixture was diluted with EtOAc (50 mL) and washed with aqueous 5% HCl (2 x 25 mL), saturated $NaHCO_3$ (1 x 25 mL) and brine (1 x 25 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to dryness *in vacuo*. The residue was applied to a 3g Biotage Samplet with minimal DCM, which was then fitted to a 25g Biotage SNAP column. The desired product was eluted with 40-100% EtOAc/Hex gradient to give ((3S,4R)-5-(3-benzoyl-5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-((4-chlorobenzyl)oxy)-4-(hydroxymethyl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (0.87 g, 1.266 mmol, 73.8 % yield) as a white foam.

[0100] N-Benzoyl protection of thymidine results in a diastereomeric mixture which gives rise to two C-5 methyl singlets and two C-6 proton singlets in a 3:2 ratio. For the α-anomer:

> ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.89 (s, 1H, diastereomer 1), 7.87 (d, *J* = 1.3 Hz, 1H, diastereomer 2), 7.67 - 7.60 (m, 1H), 7.60 - 7.39 (m, 3H), 7.39 - 7.17 (m, 5H), 6.02 (d, *J* = 8.6 Hz, 1H), 4.67 - 4.46 (m, 3H), 4.42 - 4.26 (m, 5H), 3.87 - 3.73 (m, 2H), 3.02 (s, 3H), 2.98 (s, 2H), 2.82 (p, *J* = 6.5 Hz, 1H), 2.03 (s, 3H, diastereomer 1), 1.94 (s, 3H, diasteromer 2).

((3S,4R,5R)-4-(((tert-butoxy-(2,2,2-trifluoroethoxy)dicarbonyl)amino)methyl)-3-((4-chlorobenzyl)oxy)-5-(3-benzoyl--thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (21)

[0101] In a 20 mL scintillation vial fitted with a stir bar was weighed ((3S,4R)-5-(3-benzoyl-thymidin-yl)-3-((4-chlorobenzyl)oxy)-4-(hydroxymethyl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (0.25 g, 0.364 mmol), (2,2,2-trifluoroethyl)-tert-Butyl-iminodicarbonate (0.088 g, 0.364 mmol), and triphenylphosphine (0.095 g, 0.364 mmol). The vial was charged with THF (Volume: 4 ml) and DIAD, 1.0M Solution in THF (0.364 ml, 0.364 mmol) was added dropwise. After stirring overnight, the reaction mixture was concentrated to dryness *in vacuo* and applied to a 25 g Biotage SNAP column. Product was eluted with 40-100% EtOAc/Hexanes gradient to give ((3S,4R,5R)-4-(((tert-butoxy-(2,2,2-trifluoroethoxy)dicarbonyl)amino)methyl)-3-((4-chlorobenzyl)oxy)-5-(3-benzoyl--thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (0.228 g, 0.25 mmol, 68.7 % yield) as a white foam.
1H NMR (400 MHz, Chloroform-*d*) δ 7.94 (d, *J* = 7.6 Hz, 2H), 7.63 (t, *J* = 7.4 Hz, 1H), 7.47 (t, *J* = 7.8 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 7.28 (d, *J* = 8.4 Hz, 2H), 7.15 (s, 1H), 5.99 (d, *J* = 9.2 Hz, 1H), 4.70 (d, *J* = 11.0 Hz, 1H), 4.60 (d, *J* = 10.9 Hz, 1H), 4.49 (qd, *J* = 8.3,3.4 Hz, 2H), 4.41 - 4.24 (m, 6H), 3.94 (d, *J* = 5.6 Hz, 2H), 3.20 - 3.05 (m, 1H), 2.98 (s, 2H), 2.97 (s, 4H), 1.92 (s, 3H), 1.46 (s, 9H). ESI-MS: 971 (M + Acetate)$^-$

((3S,4R,5R)-4-(((tert-butoxycarbonyl)amino)methyl)-3-((4-chlorobenzyl)oxy)-5-(thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (22)

[0102] In a 20 mL screw cap scintillation vial was ((3S,4R,5R)-4-(((tert-butoxy-(2,2,2-trifluoroethoxy)dicarbonyl)amino)methyl)-3-((4-chlorobenzyl)oxy)-5-(3-benzoyl-thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (125 mg, 0.137 mmol) weighed with a magnetic stir bar. The vial was charged with THF (Volume: 1.5 ml) and 2.0M LiOH in water (1.507 ml, 3.01 mmol), covered and allowed to stir overnight at room temperature The reaction mixture was diluted with EtOAc (7 mL) and washed with saturated sodium bicarbonate (1 x 5 mL) and brine (1 x 5 mL). The organic phase was dried over Na_2SO_4, Filtered and concentrated *in vacuo* to give ((3S,4R,5R)-4-(((tert-butoxycarbonyl)amino)methyl)-3-((4-chlorobenzyl)oxy)-5-(thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (80 mg, 0.117 mmol, 86 % yield) as an off white foam that was sufficiently pure to be used crude for subsequent reactions.
1H NMR (400 MHz, Chloroform-*d*) δ 8.61 (s, 1H), 7.36 (d, *J* = 8.4 Hz, 2H), 7.27 (d, *J* = 8.4 Hz, 2H), 7.13 (s, 1H), 6.04 (d, *J* = 9.3 Hz, 1H), 4.74 - 4.64 (m, 1H), 4.59 (d, *J* = 11.3 Hz, 1H), 4.50 (d, *J* = 11.3 Hz, 1H), 4.40 - 4.23 (m, 6H), 4.00 - 3.89 (m, 1H), 3.44 (dd, *J* = 13.6, 6.7 Hz, 1H), 3.17 (ddd, *J* = 14.3, 8.4, 5.8 Hz, 1H), 3.09 (s, 3H), 3.00 (s, 3H), 1.89 (s, 3H), 1.32 (s, 9H). ESI-MS: 681 (M)$^-$

(1R,5R,7R,8S)-tert-butyl 8-((4-chlorobenzyl)oxy)-7-(thymidin-yl)-5-(((methylsulfonyl)oxy)methyl)-6-oxa-3-azabicyclo[3.2.1]octane-3-carboxylate (23)

[0103] In a 10 mL conical reaction vial was ((3S,4R,5R)-4-(((tert-butoxycarbonyl)amino)methyl)-3-((4-chlorobenzyl)oxy)-5-(thymidin-yl)tetrahydrofuran-2,2-diyl)bis(methylene) dimethanesulfonate (60 mg, 0.076 mmol) dissolved in Tetrahydrofuran (7 ml). Sodium hydride, 60% Suspension in oil (12.21 mg, 0.305 mmol) was added to the vial at once, the vial was fitted with a stir bar and a teflon-lined septum screw-cap and the mixture was stirred at 55°C overnight. The reaction was cooled to room temperature and quenched with a few drops of MeOH added with stirring. The mixture was diluted with EtOAc (10 mL) and washed with aqueous saturated sodium bicarbonate (2 x 10 mL) and brine (1 x 10 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give a tan foam that was dissolved in a minimal amount of DCM and applied to a 1 g Biotage Samplet fitted to a 10 g Biotage SNAP column. Product was eluted with a 0-100% EtOAc/Hexanes gradient to give (1R,5R,7R,8S)-tert-butyl 8-((4-chlorobenzyl)oxy)-7-(thymidin-yl)-5-(((methylsulfonyl)oxy)methyl)-6-oxa-3-azabicyclo[3.2.1]octane-3-carboxylate (35 mg, 0.060 mmol, 78 % yield) as a white foam.

[0104] The cyclization gives a mixture of N-diastereomers in a 3:2 mixture that was unresolvable by TLC/column chromatography. This presence of the minor diastereomer gave rise to several distinct signals that are denoted by a (*). 1H NMR (400 MHz, Chloroform-*d*) δ 8.63 (s, 1H), 8.59* (s), 7.62 (s, 1H), 7.58* (s), 7.40 - 7.27 (m, 2H), 7.23 (d, *J* = 8.1 Hz, 3H), 5.80* (s), 5.79 (s, 1H), 4.66 - 4.44 (m, 2H), 4.44 - 4.27 (m, 2H), 4.09 - 3.92 (m, 2H), 3.79 (d, *J*= 12.8 Hz, 1H), 3.61* (d, *J*=12.6 Hz), 3.36 - 3.10 (m, 2H), 3.08 (s, 3H), 2.81* (s), 2.70 (s, 1H), 1.94 (s, 3H), 1.46 (s, 9H), 1.44* (s). ESI-MS: 585 $(M)^-$

1-((1R,5R,7R,8S)-8-((4-chlorobenzyl)oxy)-5-(hydroxymethyl)-6-oxa-3-azabicyclo[3.2.1]octan-7-yl)-thymidine (24)

[0105] In a 10 mL glass reaction vial was (1R,5R,7R,8S)-tert-butyl 8-((4-chlorobenzyl)oxy)-7-(thymidin-yl)-5-(((methylsulfonyl)oxy)methyl)-6-oxa-3-azabicyclo[3.2.1]octane-3-carboxylate (35 mg, 0.060 mmol) and sodium benzoate (17.21 mg, 0.119 mmol) dissolved in DMF (2 ml). The vial was fitted with a stir bar and sealed with a teflon lined screw-cap septum. The mixture was heated to 105°C in an oil bath overnight. All components had effected solution. The vial was removed from the oil bath and 10 uL removed to asses reaction completeness via TLC. White crystals started forming immediately upon cooling. TLC revealed reaction was only 50% complete, so an additional portion of sodium benzoate (17.21 mg, 0.119 mmol) was added along with 1 mL DMF to allow for stirring. The mixture was heated to 105°C for an additional 48 hours with periodic aliquots removed for TLC analysis. The thick precipitate never fully effected solution, even after heating to 105°C for two days, however the reaction went to completion with no detectable decomposition.

[0106] The reaction mixture was cooled to room temperature, diluted with EtOAc (10 mL) and washed with water (2 x 10 mL), saturated bicarbonate solution (1 x 10mL) and brine (1 x 10 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated in *vacuo*. The residue was re-dissolved in MeOH (2 ml) and sodium methoxide (6.45 mg, 0.119 mmol) was added at once. The mixture was allowed to stir overnight. TLC revealed that the reaction was complete and the mixture was concentrated to dryness. The resultant residue was re-dissolved in 1 mL of 1:1 DCM/TFA and stirred for 30 minutes at room temperature. The mixture was concentrated to dryness and applied to a 4 g RediSep Rf silica column using a minimal amount of DCM. The product was eluted with a 0-100% EtOAc/Hex gradient containing 3% TEA. The product fractions were combined and concentrated to dryness. The resultant white powder was re-dissolved in DCM (3mL) and washed with saturated bicarbonate solution (1 x 5 mL). The aqueous fraction was back extracted with 70/30 chloroform/isopropanol (2 x 5 mL). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated to give 1-((1R,5R,7R,8S)-8-((4-chlorobenzyl)oxy)-5-(hydroxymethyl)-6-oxa-3-azabicyclo [3.2.1]octan-7-yl)-thymidine (17 mg, 0.042 mmol, 69.8 % yield) as a white powder.
^{1}H NMR (400 MHz, Acetonitrile-*d*3) δ 8.05 (q, *J* = 1.2 Hz, 1H), 7.36 (s, 4H), 5.94 (s, 1H), 4.52 (dd, *J* = 38.6, 11.9 Hz, 2H), 4.14 (d, *J* = 5.1 Hz, 1H), 3.58 (dd, *J* = 33.9, 12.3 Hz, 2H), 3.09 (d, *J* = 12.7 Hz, 1H), 2.89 (d, *J* = 13.0 Hz, 1H), 2.75 (dd, *J* = 13.0, 3.2 Hz, 1H), 2.57 - 2.50 (m, 1H), 2.34 (d, *J* = 13.0 Hz, 1H), 1.98 - 1.90 (m, 2H), 1.81 (d, *J* = 1.1 Hz, 3H). ^{13}C NMR (101 MHz, CD_3CN) δ 165.01, 151.22, 138.07, 136.98, 133.72, 130.05, 129.23, 109.19, 87.42, 85.04, 73.06, 71.38, 61.04, 45.85, 43.60, 41.58, 12.71.

(1R,5R,7R,8S)-tert-butyl-8-((4-chlorobenzyl)oxy)-7-(thymidin-yl)-5-(hydroxymethyl)-6-oxa-3-azabicyclo[3.2.1]octane-3-carboxylate (25)

[0107] (1R,5R,7R,8S)-tert-butyl 8-((4-chlorobenzyl)oxy)-7-(thymidin-yl)-5-(((methylsulfonyl)oxy)methyl)-6-oxa-3-azabicyclo[3.2.1]octane-3-carboxylate (1.0 g, 1.47 mmol) and sodium benzoate (0.63 g, 4.40 mmol) were weighed into a 100 mL round bottomed flask with a stir-bar. The flask was charged with DMF (10 mL), septum sealed and heated to 100°C for 40 hours. TLC (65% EtOAc/Hex) indicated that the reaction was complete. The mixture was diluted with saturated sodium bicarbonate (100 mL) and extracted with ethyl acetate (3 x 50 mL). The organic phases were combined and washed with brine, dried over Na_2SO_4, filtered and concentrated *in vacuo* to give a tan solid that was dissolved in a mixture of dioxane (20 mL) and 2M NaOH (3 mL). The mixture was warmed to 50°C overnight. The reaction mixture was concentrated *in vacuo* to a solid and applied to a 50 g Biotage SNAP silica column and eluted using a gradient of 50-100% EtOAc in hexanes over 7 column volumes and holding at 100% EtOAc for 7 column volumes. The product containing fractions were combined and concentrated *in vacuo* to yield (1R,5R,7R,8S)-tert-butyl 8-(hydroxy)-7-(thymidin-yl)-5-(hydroxymethyl)-6-oxa-3-azabicyclo[3.2.1]octane-3-carboxylate (0.63 g, 1.24 mmol, 84.6%) as a white foam. ESI-MS: 506 (M)$^-$

(1R,5R,7R,8S)-8-Hydroxy-7-(thymidin-yl)-5-(hydroxymethyl)-(hydromethyl)-3-(2,2,2-trifluoroacetyl)-6-oxa-3-azabicyclo[3.2.1]octane (26)

[0108] (1R,5R,7R,8S)-tert-butyl 8-(hydroxy)-7-(thymidin-yl)-5-(hydroxymethyl)-6-oxa-3-azabicyclo[3.2.1]octane-3-carboxylate (0.6 g, 1.18 mmol) was dissolved in ethanol (25 mL) and transferred to a 500 mL Parr hydrogenation vessel. Pearlman's Catalyst (0.35 g) and a single drop of glacial acetic acid was added at once and the mixture was shaken on a Parr hydrogenator under a hydrogen atmosphere (40 psi) for 4 hours. TLC indicated that the reaction was complete and spot-to-spot (5% methanol in DCM). The mixture was carefully filtered through a bed of celite that was previously washed with several volumes of methanol. The celite bed was washed with ethyl acetate (100 mL) and ethanol (100 mL). The filtrate was concentrated *in vacuo* to approximately 5 mL and transferred to a 20 mL glass scintillation vial. The material was taken to dryness *in vacuo* to give an off white powder that was used without further purification.

[0109] The glass scintillation vial was fitted with a micro stir bar and charged with dichloromethane (2 mL) and trifluoroacetic acid (2 mL). The vial was sealed and set to stir for 30 minutes. The micro stir bar was removed and the volatiles removed *in vacuo*. The resultant oil was co-evaporated with toluene (2 x 4 mL), methanol (1 x 4 mL) and DCM (2 x 4 mL) to give an off white powder/residue in the vial. The residue was re-dissolved in methanol (5 mL) with a micro stir bar in the scintillation vial. Ethyl trifluoroacetate (2.00 mL, 16.9 mmol) and TEA (0.410 mL, 3.54 mmol) were added, the vial was sealed and the mixture set to stir overnight. After 20 hours, TLC of the mixture showed that the starting material was completely consumed and a new product had been formed. The volatiles were removed *in vacuo*. The residue was co-evaporated with EtOAc (2 x 5 mL) and toluene (2 x 5 mL) to give (1R,SR,7R,8D)-8-Hydroxy-7-(thymidin-yl)-5-(hydroxymethyl)-3-(2,2,2-trifluoroacetyl)-6-oxa-3-azabicyclo[3.2.1]octane (0.30 g, 79.8%) for use directly in the next tritylation step. ^{1}H NMR analysis of the crude material indicated that a mixture of diastereomers in an approximately 55:45 ratio were formed (by integration of anomeric signals).

(1R,5R,7R,8S)-8-Hydroxy-7-(thymidin-yl)-5-((4,4'-dimethoxytrityloxy)methyl)-3-(2,2,2-trifluoroacetyl)-6-oxa-3-azabicyclo[3.2.1]octane (27)

5'-O-DMTr-aCBBN(tfa)

[0110] In a 50 mL round bottomed flask, (IR,5R,7R,8S)-8-Hydroxy-7-(thymidin-yl)-5-(hydroxymethyl)-3-(2,2,2-trifluoroacetyl)-6-oxa-3-azabicyclo[3.2.1]octane (0.28 g, 0.74 mmol) was co-evaporated with pyridine (2 x 10 mL). The flask was charged with anhydrous pyridine (7 mL) and DMTr-Cl was added, at once, the solution. The flask was sealed and the mixture stirred overnight at room temperature. TLC revealed that all starting material was consumed (95% EtOAc/Hex or 5% MeOH/DCM). The reaction was quenched by addition of methanol (0.5 mL) and stirring continued for 30 minutes, followed by addition of aqueous saturated $NaHCO_3$ (30 mL). The aqueous phase was extracted with EtOAc (3 x 20 mL). The organic phases were combined and washed with brine (1 x 20 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo* to give a tan foam. The solids were dissolved in a minimum amount of DCM and applied to a 50 g Biotage silica SNAP column previously treated with 60 mL of a 25% solution of TEA in hexanes and equilibrated with 200 mL of 30% EtOAc/Hex. The product was eluted off the column with a gradient of 30-100% EtOAc in Hexanes over 10 column volumes followed by 4 column volumes of 100% EtOAc. Fractions containing pure product were combined and concentrated to give DMTr-(N-tfa)-aminoCBBN as a white foam. Both ^{1}H and ^{19}F NMR indicates two distinct diastereomers. Asterisks in the ^{1}H NMR tabulation denotes peaks where diastereomeric protons are resolved in an approximately 55:45 ratio.

^{1}HNMR(400MHz, Chloroform-*d*) δ 7.72* (d, *J* = 1.0 Hz, 1H), 7.68* (d, *J* = 1.1 Hz, 1H), 7.49 - 7.38 (m, 4H), 7.35 - 7.20 (m, 14H), 6.93 - 6.78 (m, 8H), 5.73* (s, 1H), 5.68* (s, 1H), 4.55 - 4.36 (m, 3H), 4.05* (s, 2H), 4.01* (s,2H), 3.94 - 3.84 (m, 1H), 3.79 (q, *J* = 0.7 Hz, 13H), 3.64 (t, *J* = 12.0 Hz, 1H), 3.57 - 3.38 (m, 4H), 3.38 - 3.15 (m, 4H), 2.70* (d, *J*= 3.6 Hz, 1H), 2.65* (t, *J* = 4.0 Hz, 1H), 1.47* (s, 3H), 1.41* (s, 3H), 1.28 (bs, 2H). ^{19}F NMR (376 MHz, cdcl$_3$) δ -68.61, -68.90. ESI MS: 680 (M)$^-$

(1R,5R,7R,8S)-7-(thymidin-yl)-5-((4,4'-dimethoxytriloxy)methyl)-3-(2,2,2-trifluoroacetyl)-6,8-oxa-3-azabicyclo[3.2.1]octane-8-O-(2-cyanoethyl)-N,N-diisopropylphoramidite (28)

5'-O-DMTr-aCBBN(tfa) Amidite

[0111] 5'-O-DMTr-aCBBN(tfa) (0.32 g, 0.47 mmol) was weighed in a 100 mL round-bottomed flask fitted with a stir bar. The flask was charged with dichloromethane (7 mL) and set to stir. 2-Cyanoethyl N,N,N',N'-tetraisopropylphosphordiamidite (0.283 g, 0.94 mmol) was weighed in a syringe and added at once to the solution followed by 4,5-dicyanoimidazole (55.44 mg, 0.47 mmol). The flask was immediately septum sealed and allowed to stir overnight. In process TLC at 20 hours revealed that there was only a trace of starting material, with two new spots arising that were trityl positive and appeared to char similarly to starting nucleoside when treated with Hanessian's stain following development with 5% methanol/DCM w/ UV visualization. Reaction was quenched by the addition of aqueous saturated $NaHCO_3$ solution (50 mL). The aqueous phase was extracted with ethyl acetate (4 x 20 mL). The organic phases were combined and extracted with aqueous saturated $NaHCO_3$ solution (2 x 50 mL) and brine (1 x 20 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to give a colorless oil. The crude product was dissolved in a minimum amount of DCM and applied to a 50 g Biotage silica SNAP column previously treated with 60 mL of a 25% solution of TEA in hexanes and equilibrated with 150 mL of 30% ethyl acetate/hexanes. The product was eluted off the column with a gradient of 30-100% EtOAc in Hexanes over 10 column volumes followed by 4 column volumes of 100% EtOAc. Fractions containing pure product were combined and concentrated to give DMTr-(N-tfa)-aminoCBBN amidite as a white foam. ^{31}P and ^{1}H NMR indicate the presence of four distinct products, as expected, each corresponding to a separate stereoisomer arising from the tfa protection of the cyclic amine and the phosphitylation reaction.
^{31}P NMR (162 MHz, CD3CN) δ 150.03, 149.97, 147.46. Relative intensity of 1:1:2. ^{19}F NMR (376 MHz, CD3CN) δ -69.30, -69.31, -69.47, -69.47.
ESI MS: 904.8 $(M + Na^+)^+$

Example 2: Production of 2'-C-Bridged Bicyclic Nucleoside Phosphoramidites and Conjugates

Synthesis of dimethoxytrityl (DMTr)-protected amine 2'-C-Bridsed Bicyclic Nucleoside phosphoramidite (30) as Illustrated in Figure 4A

[0112] Compound 30 was synthesized from compound 24 by subjecting compound 24 to reductive amination in the presence of formaldehyde and sodium cyanoborohydride (J. Org. Chem., 1972, 37, pp 1673-1674, Borch conditions) or similar reducing agents, such as ZnCl2/NaBH4. Following reductive methylation, the 3'-OH was unmasked by dissolving the product in ethanol and subjecting the mixture to catalytic hydrogenation using Pearlman's Catalyst in a hydrogen atmosphere (40 psi) and a trace of acetic acid. The crude reduction mixture can be directly tritylated in a cooled pyridine solution by dropwise addition of a pyridine solution of 4,4'-dimethoxytrityl chloride. The mixture was allowed to stir overnight to give the 5'-dimethoxytrityl-nuceoside which was purified by using silica gel column chromatography. The purified nucleoside was then subjected to phophitylation using 2-Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite (1.5-2equiv) and 4,5-dicyanoimidazole (1.1-2 equiv.) as a coupling catalyst in dichloromethane. The reaction was allowed to stir at least 24 hours and as long as 48 hours until quenched with saturated sodium bicarbonate solution and subjected to column chromatography to give phosphoramidite 30. Compound 30 is suitable for use in automated solid phase oligonucleotide synthesis for incorporation into a synthetic oligonucleotide.

Synthesis of an Internal Phosphoramidite Derivative of a DMTr-protected Amine 2'-C-Bridged Bicyclic Nucleoside (32) as Illustrated in Figure 4B

[0113] Compound 32 was synthesized from compound 27 in two steps by first subjecting compound 27 to alkaline hydrolysis of the trifluoroacetamide protecting group using an aqueous LiOH/THF solution. The aqueous solution was diluted with saturated sodium bicarbonate and can be extracted with ethyl acetate. The crude nucleoside can then be subjected to phosphitylation with 2-cyanoethyl phosphorodichloridite pretreated with 4-10 equivalents of anhydrous TEA or DIEA. The resultant phosphoramidite was purified via silica gel column chromatography where the silica gel was pretreated with several column volumes of 3% TEA in Hexanes prior to eluting with the appropriate mobile phase. The phosphoramidite must also be stored with a trace of TEA to prevent decomposition. Compound 32 is suitable for use in automated solid phase oligonucleotide synthesis for incorporation into a synthetic oligonucleotide. Care must be taken to use a suitable capping reagent after the coupling and oxidation steps in automated solid phase oligonucleotide synthesis. Acetic anhydride will produce a final deprotected oligonucleotide bearing an acetamide on the bicyclic nitrogen. Other activated esters or anhydrides can be used to directly make a conjugate at that bicyclic nitrogen center before proceeding with the remainder of the automated solid phase oligonucleotide synthesis.

Synthesis of DMTr-protected Fatty acid Conjugated Amine 2'-C-Bridged Bicyclic Nucleoside (33) as Illustrated in Figure 4C

[0114] Compound 33 was directly synthesized from compound 31 by utilizing a transient protection of the 3'-OH with TMS-C1 in pyridine solution. After 4 hours of reaction, the mixture was cooled in an ice bath and stearoyl chloride was added dropwise with stirring. The reaction mixture was allowed to come to room temperature overnight. The reaction mixture was quenched by addition of saturated sodium bicarbonate solution, extracted with ethyl acetate and the organics were dried. The crude material was concentrated re-dissolved in THF followed by treatment with a solution of TBAF in THF (1.25 equiv). The reaction mixture was concentrated and directly subjected to silica gel column chromatography to give compound 33. Compound 33 is amenable to phosphoramidite synthesis as described for the conversion of compound 27 to 28.

Synthesis of DMTr-Protected Sugar Conjugated Amine 2'-C-Bridged Bicylic Nucleoside as Illustrated in Figure 4D

[0115] Compound 34 was directly synthesized from compound 31 by utilizing the HBTU as the coupling reagent, 5-[(2S,3S,4R,5R,6R)-4,5-Diacetoxy-6-(acetoxymethyl)-3-acetylaminotetrahydro-2H-pyran-2-yl]valeric acid (GalNAc-C5 acid, prepared via procedure described in WO 2009073809) and at least 1 equivalent of N-methylmorpholine in DMF solution. Upon completion of the reaction (4-17 hours), the mixture was diluted with saturated bicarbonate solution and extracted with ethyl acetate. The organics were dried, the crude material concentrated and the residue was directly subjected to silica gel column chromatography to give compound 33. Compound 33 is amenable to phosphoramidite synthesis as described for the conversion of compound 27 to 28.

Example 3: Synthesis of Oligonucleotides Bearing 2'-C-Brideed Bicyclic Nucleotides

General Synthesis Methodology

[0116] Short strands of oligonucleotides bearing sugar and base modifications can be prepared once the modified nucleoside is synthesized and the free 5' and 3'-hydroxyl groups are masked with appropriate reactive groups to become a nucleotide monomer. For example, automated solid phase synthesis using phosphoramidite chemistry may be used (*see* McBride et al., Tetrahedron Letters 24:245-248 (1983) and Sinha et al., Tetrahedron Letters 24:5843-5846 (1983)). Phosphoramidite chemistry, together with related methods such as hydrogen phosphonate chemistry, has been extensively reviewed with respect to their uses in oligonucleotide chemistry (*see*, for example, Beaucage et al., Tetrahedron 48:2223-2311(1992)). During solid phase oligonucleotide synthesis, a series of nucleotide monomers are sequentially attached, via their phosphoramidite derivatives, in a predetermined order to either, depending on the direction of chain extension, the 5'-functional group or the 3'-functional group of the growing oligonucleotide strand.

[0117] The oligonucleotide strand is anchored to an insoluble moiety such as controlled pore glass or polystyrene resin beads. The method of attachment of each monomer is generally comprised of the following steps 1 through 5. Step 1 involves the protection of the reactive functionality. The common reactive functionality is the 5'-hydroxyl group of the terminal nucleoside. This functionality is usually protected with a 4,4'-dimethoxytrityl (DMT) moiety that can be removed via acid treatment. One of the features of the DMT moiety is that it forms a bright orange DMT cation during acid deprotection. This cation effectively serves as reporter group that can be monitored at a wavelength between 480 and 500 nm for the purpose of judging the completeness of the previous coupling step. Most commercially available automated synthesizers have the capability to monitor the released DMT cation. This data gives the operator an instant indication of whether or not the synthesis failed at any given step. Step 2 involves the coupling by addition of a phosphoramidite derivative and an activator. The phosphoramidite derivative is usually a nucleoside phosphoramidite. However, it may also be a phosphoramidite derivatized with a different organic moiety. Step 3 involves the capping of unreacted terminal functional groups. This step introduces an inert protective group that prevents further coupling to failure sequences. Step 4 involves oxidation of the newly formed phosphorous nucleotide backbone linkage from the trivalent phosphite to the stable pentavalent state. This oxidation step can be performed with either an oxygen-based oxidant that results in a phosphate nucleotide or a sulfurizing oxidant that results in a phosphorothioate nucleotide. Step 5 involves a repetition of the process after a washing step.

[0118] Truncated, 16 nucleotide sequence complementary to a nucleotide sequence of human miR-208a was synthesized in 1 μmol scale on a MerMade-12 automated oligonucleotide synthesis system (Bioautomation, Plano, TX, USA). The synthesizer was operated using standard detritylation, activator and capping solutions, known to those skilled in the art. Oligonucleotide chain elongation was affected using single couplings of 420 seconds for each deoxynucleotide amidite, double couplings lasting a total of 900 seconds for LNA amidites and triple couplings lasting a total of 1800 seconds for novel nucleoside amidites, such as the DMTr-aCBBN(tfa) amidite. Oxidation with either 0.025 M Iodine solution or 0.2 M PADS oxidation solution after each coupling cycle was performed to generate either phosphodiester or phosphorothioate internucleotide linkages, respectively. The unmodified anti-208a DNA sequence incorporates nine 2'-deoxythymidine residues which were selectively replaced with thymidine LNA (1T), thymidine oxoCBBN (bT), cytidine oxoCBBN (bC) or thymidine aminoCBBN (abT) nucleotides. Thymidine LNA amidite was purchased from commercial sources and matches reported spectroscopic data (*see* Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. Chem.Commun. 1998, 455-6). The Thymidyl-2'-C,4'-C-Bridged Bicyclonucleoside (thymidine oxoCBBN, bT) and cytidyl-2'-C,4'-C-Bridged Bicyclonucleoside (cytidine oxoCBBN, bC) was synthesized according to a literature procedure and all spectroscopic data matched reported values (*see* U.S. Patent No. 6,403,566, Wang,G., Girardet,J., Gunic,E. Tetrahedron 55, 1999, 7707-7724). The balance of the nucleotides was comprised of 2'-deoxynucleotides or LNA nucleotides with bases corresponding to the natural anti-208a RNA sequence. Phosphorothioate internucleotide linkages are denoted with an "s" following the base (*e.g.*, abTs or dGs), while no letter following a base indicates a phosphodiester internucleotide linkage (*e.g.*, abT or dG)

Preparation of Compound M-1 1915: dC.dT.dT.dT.dT.dT.dG.dC.abT.dC.dG.dT.dC.dT.dT.dA

[0119] Phosphoramidite Reagent **(28)** was used in the synthesis of a singly modified aminoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents and 0.025 M iodine solution. The phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle as described previously. The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55°C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 4845.2, found 4844.0 $(M)^-$.

Preparation of Compound M-1 1916: dC.dT.dT.dT.dT.abT.dG.dC.abT.dC.dG.dT.dC.dT. dT.dA

[0120] Phosphoramidite Reagent **(28)** was used in the synthesis of a double modified aminoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents and 0.025 M iodine solution. The phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle as described previously. The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55°C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 4886.2, found 4885.2 $(M)^-$.

Preparation of Compound M-1 1917: dC.dT.dT.dT.abT.abT.dG.dC.abT.dC.dG.dT.dC.dT. dT.dA

[0121] Phosphoramidite Reagent **(28)** was used in the synthesis of a triple modified aminoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents and 0.025 M iodine solution. The phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle as previously described. The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55°C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 4927.3, found 4926.1 $(M)^-$.

Preparation of Compound M-1 1918: dC.dT.dT.dT.abT.abT.dG.dC.dT.dC.dG.dT.dC.dT. dT.dA

[0122] Phosphoramidite Reagent **(28)** was used in the synthesis of a double modified aminoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents and 0.025 M iodine solution. The phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle as previously described. The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55 °C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 4886.2, found 4885.0 $(M)^-$.

Preparation of Compound M-1 1919: lCs.dTs.dTs.dTs.abTs.abTs.dGs.lCs.dTs.lCs.lGs.dTs. lCs.dTs.lTs.lA

[0123] Phosphoramidite Reagent **(28)** was used in the synthesis of the chimeric DNA/LNA/aminoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents, exchanging 0.2 M PADS in 1:1 Pyridine/ACN for the oxidizing solution. The phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle as previously described. The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55 °C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 5379.3, found 5378.3 $(M)^-$.

Preparation of Compound M-1 1920: lCs.dTs.dTs.dTs.lTs.lTs.dGs.lCs.dTs.lCs.lGs.dTs.lCs. dTs.abTs.lA

[0124] Phosphoramidite Reagent **(28)** was used in the synthesis of the chimeric DNA/LNA/aminoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents, exchanging 0.2M PADS in 1:1 Pyridine/ACN for the oxidizing solution. The phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle described above in "General Synthetic Methodology of Truncated Nucleotides". The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55 °C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 5366.3, found 5365.3 $(M)^-$.

Preparation of Compound M-10930 dC.dT.dT.dT.dT.dT.dG.dC.bT.dC.dG.dT.dC.dT.dT. dA

[0125] Thymidyl-2'-C,4'-C-Bridged Bicyclonucleoside Phosphoramidite (*see*, for example, U.S. Patent No. 6,403,566, Wang,G., Girardet,J., Gunic, E. Tetrahedron 55, 1999, 7707-7724) was used in the synthesis of a singly modified oxoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents, and 0.025 M iodine solution. All phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle as previously described. The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55°C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 4846.1, found 4845.8 (M)-

Preparation of Compound M-10924 bC.bT.bT.bT.bT.bT.dG.bC.bT.bC.dG.bT.bC.bT.bT. dA

[0126] Thymidyl-2'-C,4'-C-Bridged Bicyclonucleoside Phosphoramidite and N-Bz-Cytidyl-2'-C,4'-C-Bridged Bicyclonucleoside Phosphoramidite (*see*, for example, U.S. Patent No. 6,403,566, Wang,G., Girardet,J., Gunic,E. Tetrahedron 55, 1999, 7707-7724) was used in the synthesis of a singly modified oxoCBBN oligonucleotide. The oligonucleotide was synthesized using a Bioautomation MerMade-12 automated oligonucleotide synthesis system. The synthesis was performed according to the manufacturer's recommendations in DMT-ON mode employing commercial synthesis reagents, and 0.025 M iodine solution. All phosphoramidite reagents were added as a 0.1 M solution in acetonitrile during the appropriate coupling cycle as previously described. The cleavage of the oligonucleotide from the support was accomplished via heating of the CPG bound oligonucleotide with a solution of concentrated aqueous ammonium hydroxide at 55°C for 17 hours. The resultant aqueous solution of oligonucleotide was further purified by loading the crude DMT-ON oligonucleotide solution on a Waters Sep-Pak® Vac C18 cartridge and eluting using a standard DMT-ON oligonucleotide desalting procedure known to those knowledgeable in the art. The characterization of product was performed by HPLC-MS mass spectrometry utilizing an XBridge OST C18 2.5 um column fitted to a Waters AllianceMD HPLC with a Waters Acuity SQ Detector utilizing standard methods known to those knowledgeable in the art: calcd 5350.6, found 5350.2 (M)-

Example 4: Functional Characterizations of Oligonucleotides Bearing 2'-C-Bridged Bicyclic Nucleotides

Determination of Melting Temperature (Tm)

[0127] Melting temperature (Tm) is a critical parameter when designing synthetic oligonucleotide sequences as drugs directed towards antisense and microRNA targets. There is generally no specific Tm threshold above or below which determines activity. However, it is recognized that Tm must be significantly elevated for antisense and microRNA inhibitor oligonucleotide drugs. Furthermore, chemical modifications of the nucleotide backbones of synthetic oligonucleotide drugs (*e.g.*, phosphorothioates) are often times used to impart stability against biodegradation *in vivo*. Nevertheless, most nucleotide phosphate backbone modifications often times cause decreases in the Tm of an oligonucleotide drug duplexed with its target. Accordingly, sufficient increases in the Tm of a synthetic oligonucleotide drug against its target sequence, over that inherent in natural DNA or RNA, 2'-OMe RNA, and other similar nucleotide units, are required for the synthetic oligonucleotide drug to have sufficient specificity, target engagement and ultimately downstream regulation of cellular processes controlled by the target.

[0128] The melting temperature (Tm) of modified 16 nucleotide phosphodiester strands were determined and compared to the Tm of identical 16 nucleotide sequences having natural phosphodiester DNA nucleotides. Specifically, the relative aminoCBBN melting temperature (Tm) compared to the 2'-deoxynucleoside or oxoCBBN nucleoside with the same nucleobase was determined on a per incorporation basis by determining the difference between the melting temperature of the amino-modified 16 nucleotide length phosphodiester strand and that of the identical 16 nucleotide sequence utilizing either the 2'-deoxynucleoside or oxoCBBN phosphodiester DNA nucleotide. Tm differences of substitutions were compared only when they were placed in the same position of the sequence. Comparable values for amino-LNA and its oxo-LNA counterpart were obtained through literature references (*see* Singh, S.K., Kumar, R., Wengel J. J. Org. Chem., Vol. 63, No. 26, 1998).

[0129] For example, the modified anti-208a oligonucleotides were annealed to the complementary sequence, twenty-two nucleotides in length, comprised of RNA nucleosides and a phosphate backbone. The complementary sequence was identical to the endogenous mature miRNA. Thermal denaturation temperatures (Tm) were measured as a maximum of the first derivative plot of melting curvex (A260 vs. Temp). The duplexes were constituted at 1 μM in a 0.9% NaCl buffer. Temperature was ramped from 25 °C to 95 °C at 1 °C/min and OD's at 260 nm were read once per 30 seconds. Tm values are averages of at least two measurements.

[0130] Duplex melting temperatures for various modifications of a 16 nucleotide sequence, complementary to a nucleotide sequence of mature human miR-208a were measured using a Varian Cary 1E UV-Vis Spectrophotometer. Anti-miRNA 208a oligonucleotide sequences tested included a fully DNA phosphodiester (compound M-10931), four DNA phosphodiester oligonucleotides with 1, 2 or 3 aminoCBBN thymidine residues in place of dT residues (compounds M-11915, M-11916, M-11917, and M-11918), mixed 9 LNA/7 DNA phosphorothioate oligonucleotide (compound M-10101), and 2 mixed LNA/DNA/aminoCBBN phosphorothioate oligonucleotides where LNA thymidines of the parent compound, compound M-10101, were replaced with either 1 or 2 aminoCBBN residues (compounds M-11919 and M-1 1920). Duplexes were constituted at 1 μM in 0.9% NaCl. Temperature was ramped from 25 °C to 95 °C at 1 °C/min and OD's at 260 nm were read once per 30 seconds.

[0131] Phosphodiester oligonucleotides with aminoCBBN modifications uniformly had higher melting temperature, therefore higher affinity, towards the complimentary sequence than their fully DNA counterpart (*see* Table 3). Affinity enhancements were on the order of 5-9 °C/modification over DNA. These increases in affinity are as good as or better than literature values for LNA and aminoLNA.

Table 3

aminoCBBN, Phosphate Backbone Tm Studies, RNA Complement					
Oligo #	**Oligo Name**	**Sequence**	T_m	$\Delta T_{m,DNA}$	ΔT_m/mod
10931	208a_DNA_PO	dC;dT;dT;dT;dT;dT;dG;dC;dT;dC; dG;dT;dC;dT;dT;dA	53.1	0	**NA**
10924	208a_CBBN_C_T_DNA_ 16_3_PO	**bC**;**bT**;**bT**;**bT**;**bT**;**bT**;dG;**bC**;**bT**;**bC**; dG;**bT**;**bC**;**bT**;**bT**;dA	89.8	36.7	**2.8**
10930	208a_1CBBN_DNA_PO	dC;dT;dT;dT;dT;dT;dG;dC;**bT**;dC; dG;dT;dC;dT;dT;dA	58.3	5.3	**5.3**
11915	208a_1aminoCBBN_DNA_ PO	dC;dT;dT;dT;dT;dT;dG;dC;**abT**;dC; dG;dT;dC;dT;dT;dA	62.0	8.9	**8.9**
11916	208a_2aminoCBBN_DNA_ PO	dC;dT;dT;dT;dT;**abT**;dG;dC;**abT**; dC;dG;dT;dC;dT;dT;dA	64.6	11.5	**5.8**
11917	208a_3aminoCBBN_DNA_ PO	dC;dT;dT;dT;**abT**;**abT**;dG;dC;**abT**; dC;dG;dT;dC;dT;dT;dA	67.5	14.4	**4.8**
11918	208a_2aminoCBBN_DNA _PO_isomer	dC;dT;dT;dT;**abT**;**abT**;dG;dC;dT; dC;dG;dT;dC;dT;dT;dA	63.6	10.5	**5.2**

Table 4 Description of Notations

deoxy A	dA	oxoCBBN A	bA
deoxy G	dG	oxoCBBN G	bG
deoxy C	dC	oxoCBBN C	bC
deoxy T	dT	OxoCBBN T	bT
lna A	lA	aminoCBBN A	abA
lnaG	lG	aminoCBBN G	abG
lna C	lC	aminoCBBN C	abC
lna T	lT	aminoCBBN T	abT
deoxy A P=S	dAs		
deoxy G P=S	dGs		
deoxy C P=S	dCs		
deoxy T P=S	dTs		
lna A P=S	lAs		
lnaG P=S	lGs		
lna C P=S	lCs		
lna T P=S	lTs		

[0132] Comparison of the aminoLNA-T to its oxo-analogue, LNA-T, reveals that aminoLNA-T is less stabilizing toward its complement than LNA-T. Similarly, aminoENA-T appears to have very little duplex stabilizing effect over that of its oxo-analogue. Surprisingly, comparison of the aminoCBBN-T to its oxoCBBN-T analogue shows that the aminoCBBN modification is significantly more stabilizing than oxoCBBN-T by 2-4 °C/ modification (*see* Tables 5 and Figure 5). Without wishing to be bound by theory, it is postulated that the 2'-O of LNA, a proton acceptor, has a more stabilizing effect towards duplex hydration and stability than when it is replaced by a proton donor at the 2'-position as in the case of aminoLNA. Conversely, aminoCBBN appears to have a much more positive effect on duplex hydration and stability than its oxoCBBN analogue and offers Tm enhancements not seen in any other 2'4'-Carbon-Bridged Bicyclic Nucleotides. (*see* Figure 5).

Table 5

aminoCBBN, PS Backbone 10101-like Tm Studies, RNA Complement					
Oligo #	**Oligo Name**	**Sequence**	T_m	$\Delta T_{m,parent}$	ΔT_m/mod
10101	208a_10101	lCs;dTs;dTs;dTs;lTs;lTs;dGs; lCs;dTs;lCs;lGs;dTs;lCs;dTs; lTs;lA	86.7	NA	NA
11919	208a_10101_1aminoCBBN_P_S	lCs;dTs;dTs;dTs;lTs;lTs;dGs; lCs;dTs;lCs;lGs;dTs;lCs;dTs; **abTs**;lA	80.04	-6.66	-6.66
11920	208a_10101_2minoCBBN_PS	lcs;dTs;dTs;dTs;**abTs**;**abTs**; dGs;lCs;dTs;lCs;lGs;dTs;lCs; dTslT;lA	85.125	-1.575	-0.7875
amino-Nucleoside, Phosphate Backbone Tm Studies, RNA Complement					
	DNA_9mer_ PO_ 3LNA-T	dG;**lT**;dG;dA,**lT**;dA;**lT**;dG;dC	50	NA	NA
	DNA_9mer_ PO_ 3aminoLNA-T	dG;**alT**;dG;dA;**alT**;dA;**alT**;dG; dC	47	**-1**	**-1**
10930	208a_1CBBN_DNA PO	dC;dT;dT;dT;dT;dT;dG;dC;**bT**; dC;dG;dT;dC;dT;dT;dA	58.3	**NA**	**NA**
11915	208a_1aminoCBBN_DNA_PO	dC;dT;dT;dT;dT;dT;dG;dC;abT; dC;dG;dT;dC;dT;dT;dA	62.0	**+3.7**	**+3.7**

Cell Culture Activity of anti-208a Oligonucleotides

[0133] A HeLa cell line stably expressing miR-208a was generated. Specifically, a miRNA expression vector (Cell BioLabs, Inc.) expressing miR-208a was transfected into HeLa cells. Cells were then selected using a puromycin selection screen and clones which had detectable miR-208a expression as measured by qPCR were isolated (Ct value = ~30).

[0134] The cells were plated in a black-walled 96 well plate with 10,000 cells per well. After twenty-four hours following plating, the cells were transfected with a dual-luciferase plasmid containing the miR-208a binding site in the 3' UTR of the renilla gene and various miR-208a inhibitors (compounds M-11919, M-11920, and M-10101). Compound M-10591 was a non-targeting control. The cells were incubated for 24 hours at 37°C and then both firefly (as a transfection normalization) and renilla levels were measured by luminescence using the Dual-Luciferase Reporter Assay System (Promega). Data was normalized to cells treated with only the miR-208a dual luciferase plasmid (psi check 208a). The psi check 2 cells were treated with a dual luciferase plasmid that does not include a miR-208a binding site.

[0135] Results demonstate that compound M-11920 has comparable activity as compound M-10101, which is an optimized miR208a inhibitor that includes only LNA/DNA bases (*see* Figure 6). Accordingly, multiple replacements of LNA residues with aminoCBBN residues result in full retention of miR208a inhibition activity. Compound M-11919 has slightly less activity compared to the other two inhibitors (*see* Figure 6). The activity of compound M-11919 correlates with the Tm data which shows that compound M-11919 has less affinity for the miR-208a RNA than the M-11920 compound.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

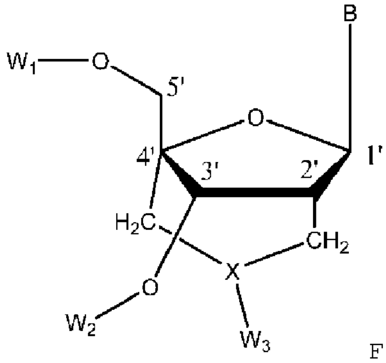
- US 8642751 B **[0012]**
- WO 2012061810 A **[0026] [0031]**
- WO 2012083005 A **[0028]**
- US 20130345288 A **[0028]**
- US 6268490 B **[0037]**
- US 6316198 B **[0037]**
- US 6403566 B **[0037] [0038] [0118] [0125] [0126]**
- US 6770748 B **[0037]**
- US 6998484 B **[0037]**
- US 6670461 B **[0037]**
- US 7034133 B **[0037]**
- US 6833361 B **[0038]**
- WO 2009012468 A **[0048]**
- WO 2007070483 A **[0048]**
- WO 20071070483 A **[0048]**
- WO 2009062169 A **[0048]**
- WO 2010135570 A **[0048]**
- WO 2008016924 A **[0048]**
- WO 2009018492 A **[0048]**
- US 60950565 B **[0048]**
- WO 2009058818 A **[0048]**
- US 61047005 B **[0048]**
- WO 2009018493 A **[0048]**
- WO 2010120969 A **[0048]**
- US 20090226375 A **[0048]**
- WO 2010129950 A **[0048]**
- US 2012059349 W **[0048]**
- WO 2010019574 A **[0048]**
- WO 2009117418 A **[0048]**
- WO 2009105759 A **[0048]**
- WO 2012148373 A **[0048]**
- WO 2011153542 A **[0048]**
- US 20100324118 A1 **[0048]**
- US 20120238619 A1 **[0048]**
- WO 2012153135 A **[0048]**
- US 20110281933 A1 **[0048]**
- US 7404969 B **[0053]**
- US 7202227 B **[0053] [0057]**
- WO 2010129672 A **[0054]**
- US 5981505 A **[0057]**
- US 6217900 B **[0057]**
- US 6383512 B **[0057]**
- US 5783565 A **[0057]**
- US 6379965 B **[0057]**
- US 6127170 A **[0057]**
- US 5837533 A **[0057]**
- US 6747014 B **[0057]**
- WO 2003093449 A **[0057]**
- US 6416510 B **[0059]**
- US 6716196 B **[0059]**
- US 6953466 B **[0059]**
- WO 2005082440 A **[0059]**
- WO 2006089340 A **[0059]**
- US 20070203445 A **[0059]**
- US 20060148742 A **[0059]**
- US 20070060907 A **[0059]**
- WO 2009073809 A **[0115]**

Non-patent literature cited in the description

- **CARUTHERS et al.** New Chemical Methods for Synthesizing Polynucleotides. *Nucleic Acids Symp. Ser.*, 1980, vol. 7, 215-23 **[0051]**
- Remington's Pharmaceutical Sciences. 1035-1038, 1570-1580 **[0063]**
- *J. Org. Chem.*, 1972, vol. 37, 1673-1674 **[0112]**
- **MCBRIDE et al.** *Tetrahedron Letters*, 1983, vol. 24, 245-248 **[0116]**
- **SINHA et al.** *Tetrahedron Letters*, 1983, vol. 24, 5843-5846 **[0116]**
- **BEAUCAGE et al.** *Tetrahedron*, 1992, vol. 48, 2223-2311 **[0116]**
- **SINGH, S. K. ; NIELSEN, P. ; KOSHKIN, A. A. ; WENGEL, J.** *Chem.Commun.*, 1998, 455-6 **[0118]**
- **WANG,G. ; GIRARDET,J. ; GUNIC,E.** *Tetrahedron*, 1999, vol. 55, 7707-7724 **[0118] [0126]**
- **WANG,G. ; GIRARDET,J. ; GUNIC, E.** *Tetrahedron*, 1999, vol. 55, 7707-7724 **[0125]**
- **SINGH, S.K. ; KUMAR, R. ; WENGEL J.** *J. Org. Chem.*, 1998, vol. 63 (26 **[0128]**

P a t e n t k r a v

1. Oligonukleotid omfattende mindst et 2'-C-forbundet bicyklisk nukleotid, hvor det 2'-C-forbundne bicykliske nukleotid har strukturen med formel I:



Formel I

hvor

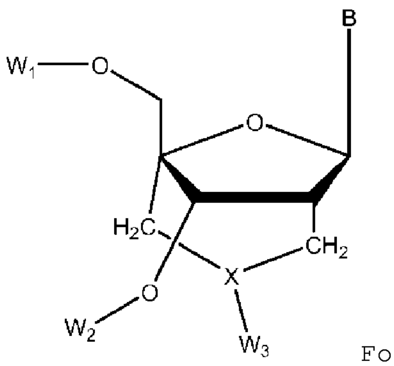
X er N;

W_1 og W_2 hver uafhængigt er udvalgt blandt H, en alkoholbeskyttelsesgruppe, phosphatester, phosphorothioatester, di- eller tri-phosphat, phosphoramidit eller en eller flere nukleotid(er) på 5'- eller 3'-siden;

W_3 uafhængigt er udvalgt blandt nul, H, O, en amin-beskyttelsesgruppe, phosphoramidit, phosphoramidatester, phosphordiamidatester, methyl, alkyl, cycloalkyl, carboxamid, et sukker, en fedtsyre, et andet molekylært konjugat, $-C_{1-4}(O)R$ eller -COOR, hvor R er aryl, lineær eller cyklisk alkyl eller alkenyl, sukker, fedtsyre eller et andet molekylært konjugat, såsom et lægemiddelkonjugat; og

B er en nukleobase.

2. 2'-C-forbundet bicyklisk nukleosid eller nukleotid med strukturen med formel I:



Formel I

hvor

X er N;

W_1 og W_2 hver uafhængigt er udvalgt blandt H, en alkoholbeskyttelsesgruppe, phosphatester, phosphorothioatester, di- eller tri-phosphat eller phosphoramidit;
W_3 uafhængigt er udvalgt blandt H, en amin-beskyttelsesgruppe, phosphoramidit, phosphoramidatester, phosphordiamidatester, methyl, alkyl, cycloalkyl, carboxamid, et sukker, en fedtsyre, et andet molekylært konjugat, -C_{1-4}(O)R eller -COOR, hvor R er aryl, lineær eller cyklisk alkyl eller alkenyl, sukker, fedtsyre eller et andet molekylært konjugat, såsom et lægemiddelkonjugat; og
B er en nukleobase.

3. Oligonukleotid ifølge krav 1, hvor alkoholbeskyttelsesgruppen er udvalgt blandt 4,4'-dimethoxytrityl, acetyl, silyl eller syrelabil ether.

4. Oligonukleotid ifølge krav 1 eller 3, hvor amin-beskyttelsesgruppen er udvalgt blandt carbobenzyloxy (Cbz), p-methoxybenzyl-carbonyl (Moz eller MeOZ), tert-butyloxycarbonyl (BOC), 9-fluorenylmethyloxycarbonyl (FMOC), acetyl (Ac), benzoyl (Bz), benzyl (Bn) eller trifluoracetyl (tfa).

5. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-4, hvor nukleobasen er en purinbase.

6. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-5, hvor nukleobasen er en pyrimidinbase.

7. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-6, som har fra 5 til 9 2'-C-forbundne bicykliske nukleotider.

8. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-7, hvor mindst 25 % af nukleotiderne er 2'-C-forbundne bicykliske nukleotider.

9. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-8, hvor oligonukleotidet er fra ca. 5 til 50 nukleotider langt.

10. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-9, hvor nukleotidet omfatter mindst et nukleotid udvalgt blandt 2'-deoxy, 2'-O-methyl, 2'-

fluor eller en 2'- til 4'-methoxy-brostruktur.

11. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-10 omfattende en eller flere phosphorothioat-bindinger.

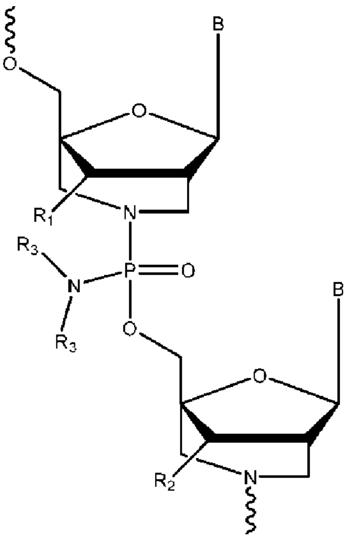
12. Oligonukleotid ifølge krav 11, hvor oligonukleotidet er fuldstændig phosphorothioat-bundet.

13. Oligonukleotid ifølge krav 11, omfattende en til tre phosphat-bindinger.

14. Oligonukleotid ifølge et hvilket som helst af kravene 11-13, hvor de 2'-C-forbundne bicykliske nukleotider er phosphorothioat-bundne.

15. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-10, hvor de 2'-C-forbundne bicykliske nukleotider er phosphorodiamidat-bundne.

16. Oligonukleotid ifølge krav 15, hvor phosphorodiamidat-bindingen er vist som



hvor

R_1, R_2 og R_3 hver uafhængigt er udvalgt blandt H, alkyl, alkenyl, oxo, aryl, benzyl, halogen, -OH, -NH2, alkoxy, en alkoholbeskyttelsesgruppe eller en amino-beskyttelsesgruppe; og

B er en nukleobase.

17. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-16, omfattende mindst en purin- og/eller pyrimidinbase-modifikation.

18. Oligonukleotid ifølge krav 17, hvor base-modifikationen er en carboxamido.

19. Oligonukleotid ifølge krav 17, hvor base-modifikationen er en carboxamido-del ved C-8-positionen for purin eller C-5-positionen for pyrimidin.

20. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-19 omfattende en eller flere morpholino-nukleotider.

21. Oligonukleotid ifølge et hvilket som helst af kravene 1 eller 3-20, omfattende en nukleotidsekvens, der er i det væsentlige komplementær til en nukleotidsekvens af human mikroRNA.

DRAWINGS

FIGURE 1

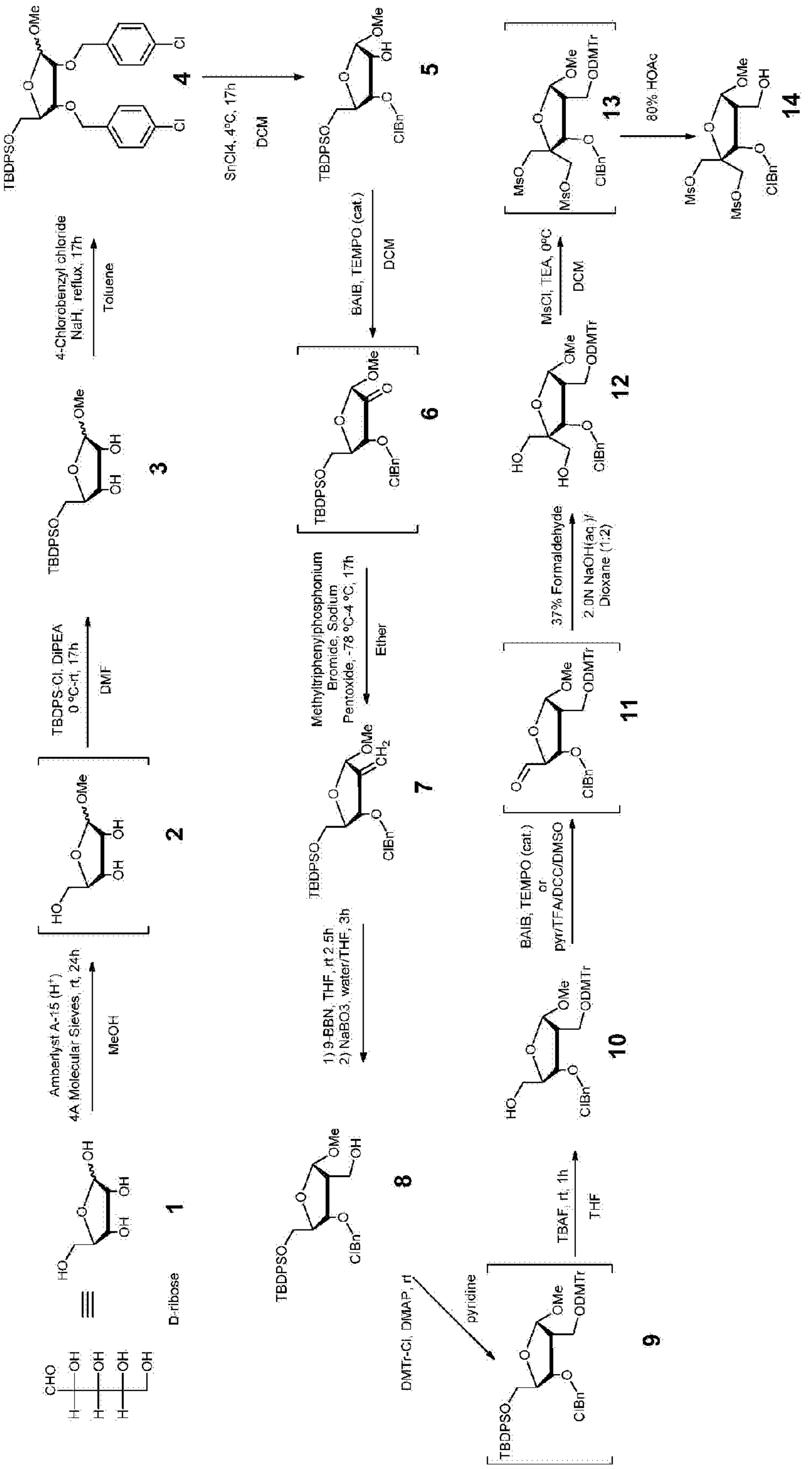


FIGURE 1 (Continued)

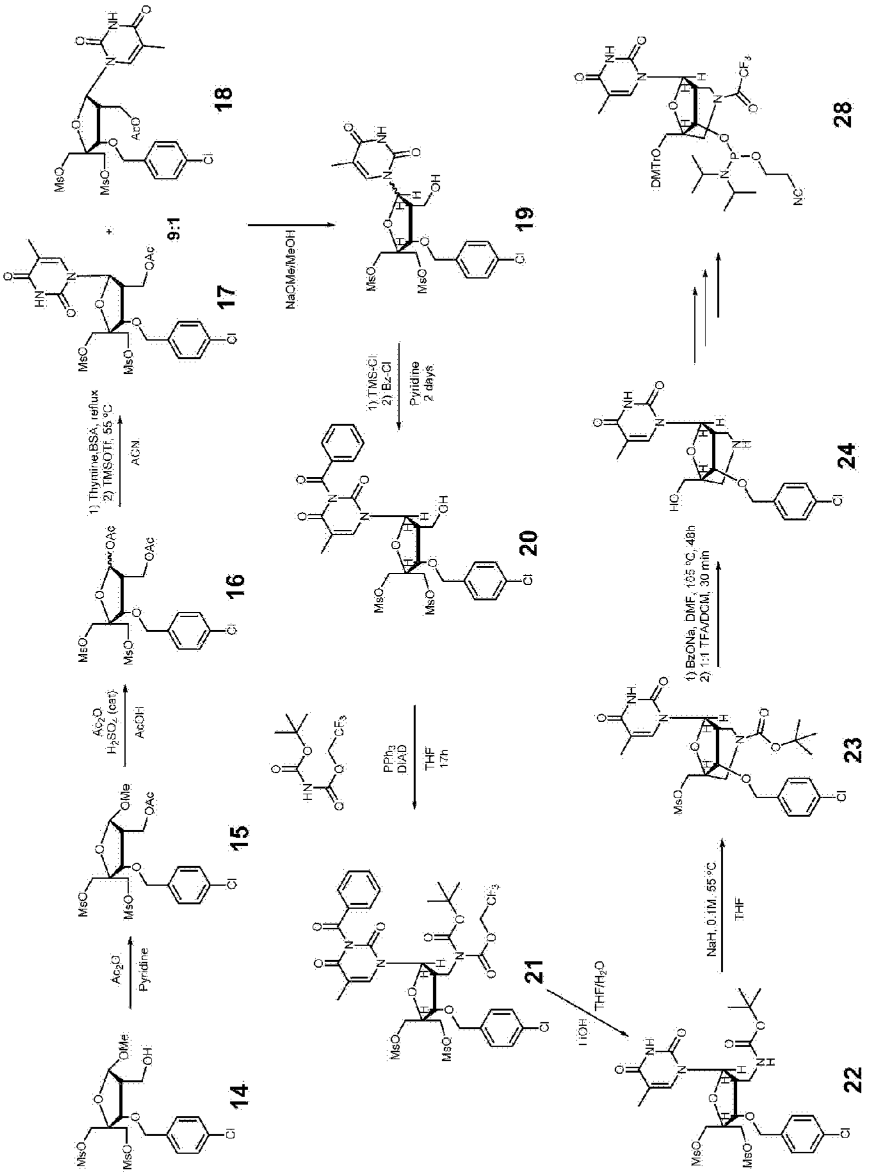


FIGURE 1 (Continued)

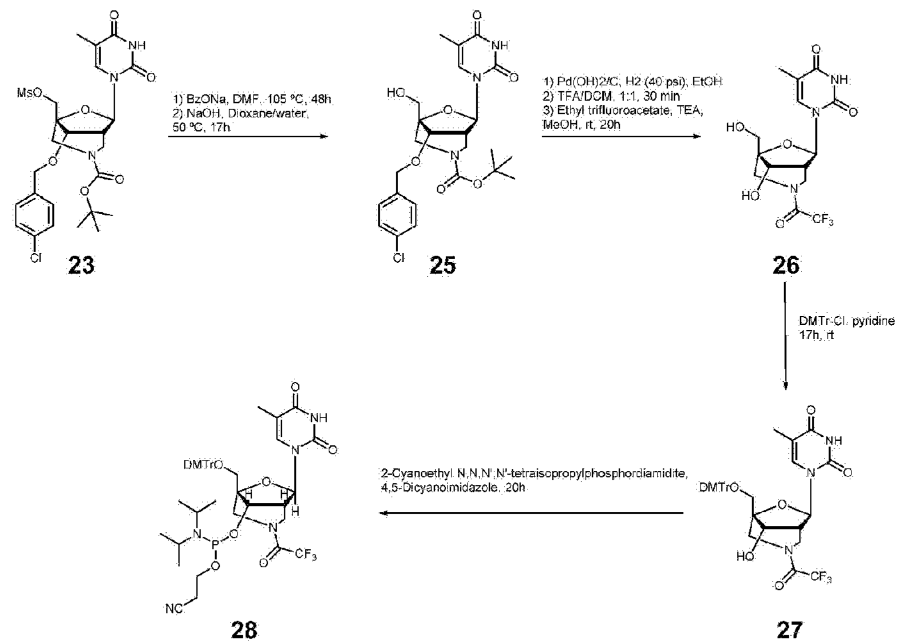


FIGURE 2

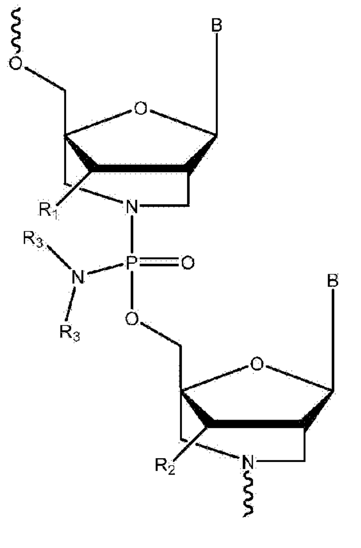


FIGURE 3A

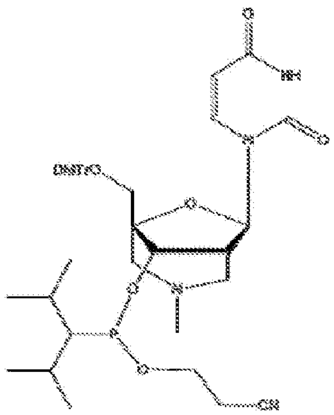


FIGURE 3B

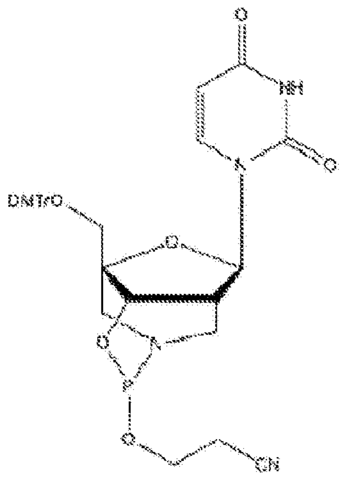


FIGURE 3C

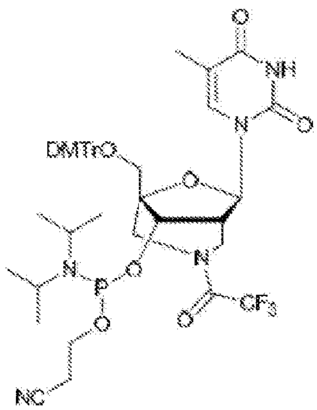


FIGURE 3D

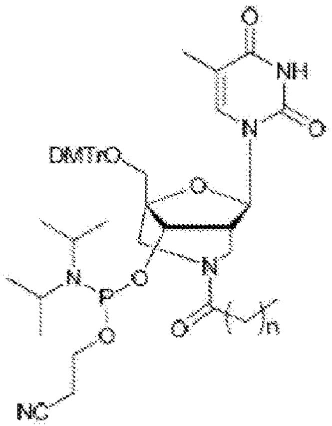


FIGURE 3E

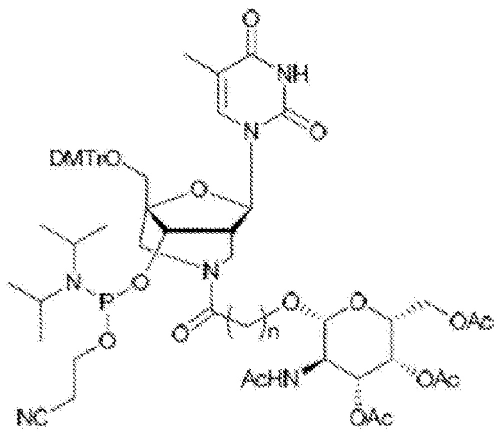


FIGURE 4A

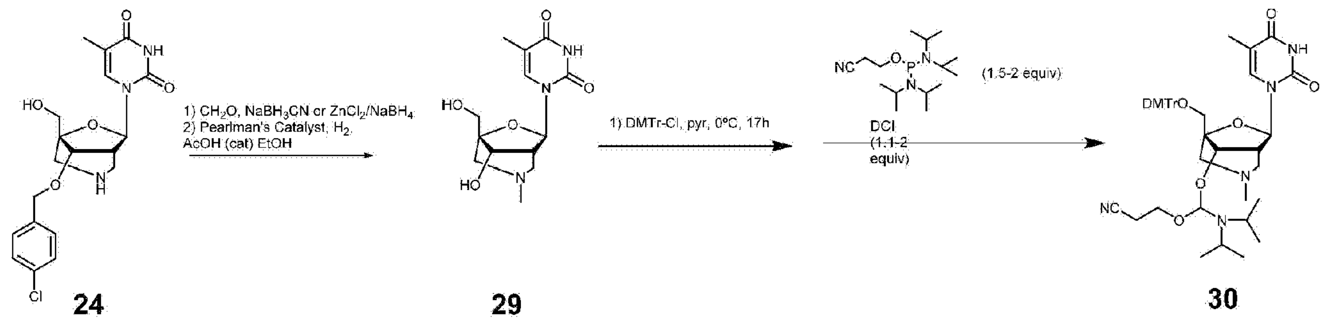


FIGURE 4B

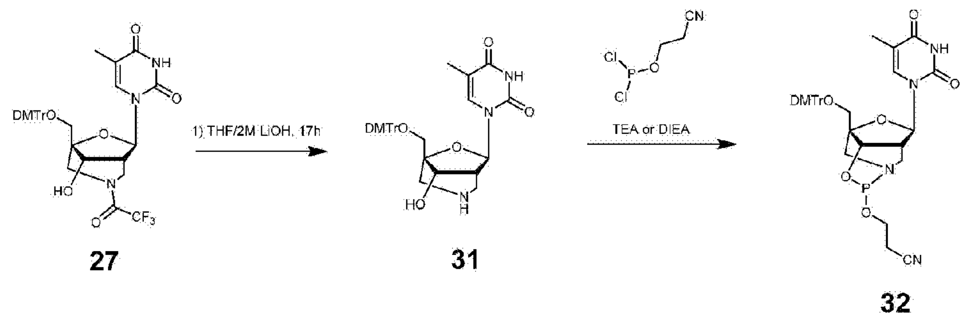
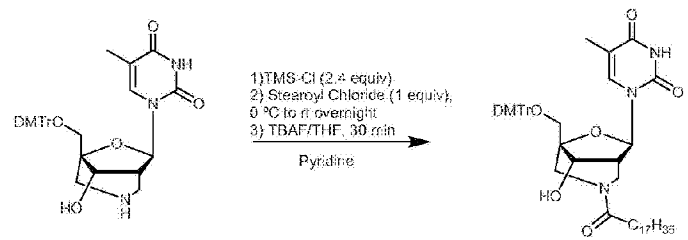
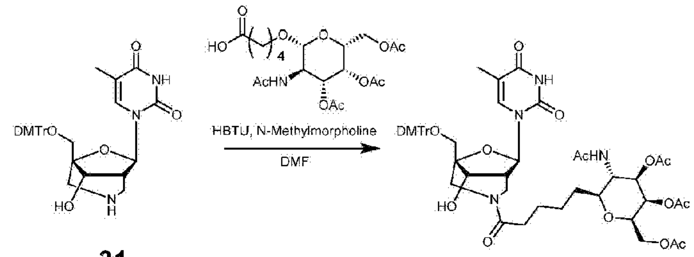


FIGURE 4C



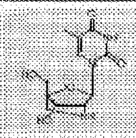
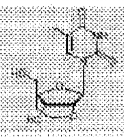
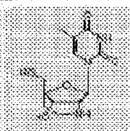
31 33

FIGURE 4D



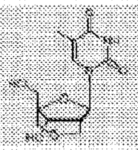
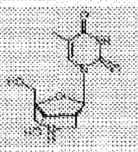
31 34

FIGURE 5A

Modification	LNA	aminoLNA	oxoENA	aminoENA
Structure				
ΔT_m, PO BB, RNA Complement, vs. dT Single Mod	+9 °C/Mod*	+6.6 °C/Mod*	NA	+2.5-4.0 °C/Mod#
ΔT_m, PO BB, RNA Complement, vs. dT Multiple Mods	+5-7 °C/Mod*	+6.3 °C/Mod*	+3.5-5.2 °C/Mod**	NA

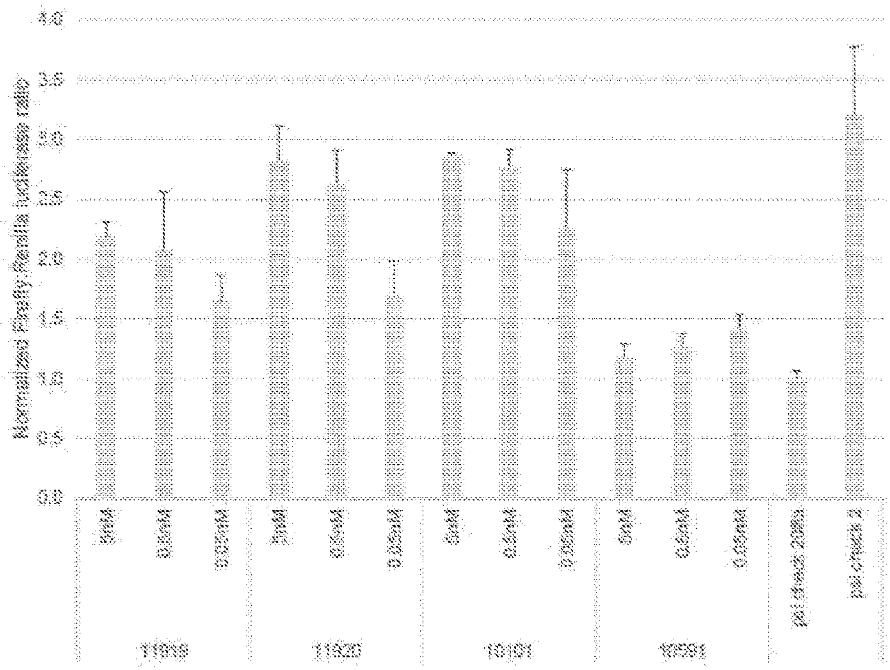
*Literature value, 9-mer
**Literature value, 12-mer

FIGURE 5B

Modification	oxoCBBN	aminoCBBN
Structure		
ΔT_m, PO BB, RNA Complement, vs. dT Single Mod	+5.3 °C/Mod##	+8.9 °C/Mod##
ΔT_m, PO BB, RNA Complement, vs. dT Multiple Mods	+2.8 °C/Mod##	+5.2 °C/Mod##

#Literature value, 15-mer
##miRagen experimental value, 16-mer

FIGURE 6


Normalized Firefly:Renilla luciferase ratio
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5nM
0.5nM
0.05nM
5nM
0.5nM
0.05nM
5nM
0.5nM
0.05nM
5nM
0.5nM
0.05nM
11919
11920
10101
10591