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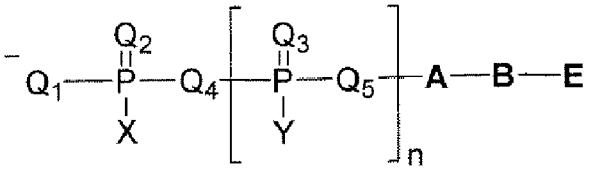
(54) **Title**: SYNTHETIC METHODS AND DERIVATIVES OF TRIPHOSPHATE OLIGONUCLEOTIDES



(I)

(57) **Abstract**: The invention features a oligonucleotide of formula (I) or pharmaceutically acceptable salts, or prodrugs thereof: (I) which are capable of inducing an anti-viral or an antibacterial response, in particular, the induction of type I IFN, IL-18 and/or IL-1β by binding to RIG-1. The invention relates to methods of making and using modified oligonucleotide comprising at least one triphosphate or analogs thereof. The invention further relates to methods for treating various disorders and diseases such as viral infections, bacterial infections, parasitic infections, tumors, allergies, autoimmune diseases, immunodeficiencies and immunosuppression.

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Synthetic methods and derivatives of triphosphate oligonucleotides

TECHNICAL FIELD

The invention relates to methods of making and using modified nucleic acid comprising at least one triphosphate or analogs thereof. This invention also relates to said iRNA agents that are modified so as to either stimulate or inhibit the immune system of a subject. This invention describes the use of iRNA and RNA agents which are capable of inducing an anti-viral or an antibacterial response, in particular, the induction of type I IFN, IL-18 and/or IL-1β by modulating RIG-I. This invention also relates to the use of iRNA agents that sequence-specifically target the mRNA of certain immune-related and diseases such as viral infections, bacterial infections, parasitic infections, tumors, allergies, autoimmune diseases, immunodeficiencies and immunosuppression.

BACKGROUND

Double-stranded RNA molecules (dsRNA) can block gene expression by virtue of a highly conserved regulatory mechanism known as RNA interference (RNAi). Briefly, the RNA III Dicer enzyme processes dsRNA into small interfering RNA (siRNA) of approximately 22 nucleotides. The siRNA is assembly into a protein/RNA complex called RNA-induced silencing complex (RISC; Bartel, *Cell*, **2004**, *116*, 281-297). One of the strands of the siRNA duplex, the antisense strand or guide strand, hybridizes to a messenger RNA (mRNA) through formation of the specific Watson–Crick base pairs. The endonuclease component of the RISC, Slicer, cleaves the targeted mRNA (Meister *et al.*, *Mol. Cell*, **2004**, *15*, 185-197.). The antisense strand of the siRNA duplex is not cleaved or otherwise degraded in this process, and the RISC that includes this antisense strand can subsequently cleave additional complementary mRNAs.

Many diseases (*e.g.*, cancers, hematopoietic disorders, endocrine disorders, and immune disorders) arise from the abnormal or otherwise unwanted expression or activity of a particular gene or group of genes. For example, disease can result through misregulated gene expression, expression of a mutant form of a protein, or expression of

viral, bacterial or other pathogen-derived genes. The RNAi pathway can be used to inhibit or decrease the unwanted expression of such genes (Agrawal *et al.*, *Microbiol Mol Biol Rev.*, **2003**, *67*, 657-685; Alisky & Davidson, *Am. J. Pharmacogenomics*, **2004**, *4*, 45-51).

A number of receptor proteins have evolved that take part in nucleic acid recognition. Recent studies indicate that one of the most important protein receptors for antiviral defense is the retinoic-acid-inducible protein I (RIG-I), a member of the helicase family containing two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain (M. Yoneyama et a/., Nat Immunol 5, 730 (JuI, 2004)). RIG-I-mediated recognition of a specific set of RNA viruses (flaviviridae, paramyxoviridae, orthomyxoviridae and rhabdoviridae) (M. Yoneyama et a/., Nat Immunol 5, 730 (JuI, 2004); R. Sumpter, Jr. et al., J Virol 79, 2689 (Mar, 2005); H. Kato et a/., Nature 441 , 101 (Apr 9, 2006)) has a critical role in antiviral host defense in vitro and in vivo. A second member of the the helicase family, MDA-5, is responsible for the antiviral defense against a reciprocal set of RNA viruses (picomaviridae)(H. Kato et al., Nature 441 (7089): 101 -105, Apr 9, 2006).

In addition to RIG-I and MDA-5, the four members of the Toll-like receptor (TLR) family, TLR3, TLR7, TLR9 and TLR9, are also known to be involved in viral nucleic acid recognition. RIG-I and MDA-5 differ from the TLRs in their subcellular localization, expression patem, signal transduction pathways and ligands.

While RIG-I and MDA-5 are cytosolic receptors, TLR3, TLR7, TLR8 and TLR9 are located in the endosomal membrane.

While TLRs are mainly expressed on certain defined immune cell subsets (i.e. TLR9 restricted to PDC and B cells), RIG-I and MDA-5 are expressed in both immune and nonimmune cells (H. Kato et al., Immunity 23, 19 (JuI_1 2005)).

Besides distinct expression profiles and cellular localization, signalling of endosomal TLRs and the two cytoplasmic receptors RIG-I and MDA-5 differs. While TLR3 signals via TRIF and TLR7, TLR8 and TLR9 signal via MyD88, RIG-I recruits a CARD-containing adaptor.

The iRNA agents described in this patent are duplexes of chemically synthesized oligoribonucleotides. One or both strands of the duplex may be chemically modified to

alter the properties of the duplex as described below. In addition to RISC-mediated cleavage of mRNA recognized in a sequence specific manner, the duplexes may have an effect on the immune system. This invention relates to iRNA agents that are modified so as to either stimulate or inhibit the immune system of a subject. These iRNA agents may have a dual function and also sequence-specifically target an mRNA of a gene not related to the immune cascade. These immune stimulatory or inhibitory iRNA agents may also be used in conjunction with a second therapeutic, which may be a chemotherapeutic agent, an antibiotic, or a second iRNA agent. This invention also relates to the use of iRNA agents that sequence specifically target the mRNAs of certain immune-related proteins, for example TLR 3, TLR 7, TLR 8 and TLR 9, for degradation via a RISC-based mechanism.

Pathogen recognition is mediated by the innate recognition arm of the immune system. Unlike the adaptive immune system, innate immunity does not have the potential for recognition of all possible antigens. Instead, a few highly conserved structures present in many different microorganisms are recognized. The innate immune response is based on the recognition of ligands by pathogen recognition receptors (PRRs) on epithelial and immune cells. The ligands include lipopolysaccharide (LPS) from the gram-negative cell wall, peptidoglycan, lipotechoic acids from the gram-positive cell wall, mannose sugar, bacterial DNA, N-formylmethionine, double-stranded RNA from viruses, and glucans from fungal cell walls. Innate immunity is activated immediately or within several hours after exposure to a recognized ligand.

Cells involved in the innate immune response include phagocytic cells, basophils, mast cells, eosinophils, and natural killer cells (NK cells). There are two functionally different classes of cell surface pattern-recognition receptors on these cells: endocytic pattern-recognition receptors and signaling pattern-recognition receptors. For the purpose of this document, signaling pattern-recognition receptors, in general, and the toll-like receptors, in particular, are most important. The toll-like receptors (TLRs) bind several different molecules of microbial origin including DNA from bacteria and double-stranded RNA (Janssens & Beyaert, *Clin. Microbiol. Rev.*, **2003**, *16*, 637-646).

When a ligand binds to a TLR, a signal is transmitted to the nucleus and genes coding for the synthesis of intracellular regulatory molecules are expressed (Ulevitch,

Nat. Rev. Immunol., **2004**, *4*, 512-520). TLR signaling relies on adaptor proteins including MyD88, Tollip, Mal, and others. These adaptor proteins activate cellular responses to induce production of inflammatory cytokine production, induce maturation of dendritic cells, and induce production of interferons. The cytokines, in turn, trigger innate immune defenses such as inflammation, fever, and phagocytosis and provide an immediate response against the invading microorganism. TLRs also participate in adoptive immunity by triggering various secondary signals needed for humoral immunity (the production of antibodies) and cell-mediated immunity (the production of cytotoxic T-lymphocytes and additional cytokines).

The iRNA agents described in this document may activate the innate immune system through one of the receptors known to bind nucleic acids or nucleotide analogs. Toll-like receptor 3 (TLR3) is the receptor for double-stranded RNA. TLR3 is expressed on dendritic cells, fibroblasts, macrophages, and epithelial cells (Matsumoto *et al.*, *Microbial. Immunol.*, **2004**, *48*, 147-154). The adaptor molecule for TLR3 is TICAM-1. Binding of TLR3 to TICAM-1 induces multiple signaling cascades that ultimately lead to production of type I interferons (IFN-αβ, Matsumoto *et al.*, *Microbial. Immunol.*, **2004**, *48*, 147-154). The interferons are cytokines that induce uninfected cells to produce enzymes capable of degrading RNA thus preventing viral replication. Interferons also activate a variety of cells important to defense including cytoxic T-lymphocytes, macrophages, and NK cells.

Single-stranded RNA recognition is mediated in mouse by Toll-like receptor 7 and in humans by TLR-8. In mice, TLR7 binds to the adaptor MyD88 and leads to activation of IFN-α. Diebold *et al.* (*Science*, **2004**, *303*, 1529-1531) showed that influenza virus RNA, polyuridylic acid, and in vitro synthesized mRNA all induced production of IFN-α in plasmacytoid dendritic cells. Heil *et al.* (*Science*, **2004**, *303*, 1526-1529) showed that guanine- and uridine-rich RNA oligonucleotides of 20 residues with phosphorothioate termini stimulated dendritic cells and macrophages to secrete INF-α and proinflammatory and regulatory cytokines. Using TLR-deficient mice, they showed that mouse TLR-7 and human TLR-8 were responsible for binding to single-stranded RNA. Human TLR-7 is also activated by guanine nucleotide analogs (Lee *et al.*, *Proc. Natl. Acad. Sci.* USA, **2003**, *100*, 6646-6651).

DNA from bacteria has stimulatory effects on mammalian immune cells. This response depends on the presence of unmethylated CpG dinucleotides in the bacterial DNA; mammalian DNA has a low frequency of CpG dinucleotides and these are mostly methylated, therefore, mammalian DNA does not have immunostimulatory activity. The cellular response to CpG DNA is mediated by TLR9 (Hemmi *et al.*, *Nature*, **2000**, *408*, 740-745). Short DNA oligonucleotides with a CpG motif have immune stimulatory effects that depend on the bases flanking the CpG dinucleotide, on the number and spacing of the CpG motifs, on the presence of poly G sequences in the ODN, and on the ODN backbone (Krieg *et al.*, *Nature*, **1995**, *374*, 546-549; Krieg, In: *Antisense Drug Technology: Principles, Strategies, and Applications*, **2001**, ed. Crooke, S.T., pp. 471-516, Marcel Dekker, Inc., New York).

Double-stranded RNA may also activate the innate immune system through interaction with a ubiquitously-expressed serine/threonine protein kinase called PKR. PKR is part of the TLR4 cascade activated by TLR4 binding of bacterial LPS. PKR is induced by interferon and activated by dsRNA, cytokines, growth factors, and stress signals. PKR is autophosphorylated and activated upon binding to dsRNA. Activation results in inhibition of protein synthesis via the phosphorylation of eIF2a and also induces transcription of inflammatory genes by PKR-dependent signaling of the activation of different transcription factors (Williams, *Oncogene*, **1999**, *18*, 6112-6420). PKR up-regulates NF-κB expression through phosphorylation of its inhibitor IkB (Kumar *et al.*, *Proc. Natl. Acad. Sci. USA*, **1994**, *91*, 6288-6292). As few as 11 base pairs of dsRNA can bind to PKR and induce activity, but maximal activation requires at least 30 base pairs (Manche *et al.*, *Mol. Cell Biol.*, **1992**, *12*, 5238-5248; Nanduri *et al.*, *EMBO J.*, **1998**, *17*, 5458-5465). Interestingly, Kim *et al.* (*Nat. Biotechnol.*, **2004**, *22*, 321-325) recently showed that the 5' triphosphate on siRNAs transcribed using T7 RNA polymerase was responsible for type I IFN induction.

There have been reports that siRNA duplexes are able to trigger an immune response in human cells under certain conditions. Sledz *et al* (*Nat. Cell Biol.*, **2003**, *5*, 834-839) reported induction of the interferon system with each of the six different siRNA duplexes tested. Bridge *et al.* (*Nat. Genet.*, **2003**, *34*, 263-264) reported that some, but not all, of the hairpin siRNAs delivered using viral vectors induced expression of an

interferon-stimulated gene. The concentration of the siRNA duplex is directly correlated with the extent of the immune response. These two reports showed that the siRNA duplexes activated PKR. In contrast, data from Kariko *et al.* (*Cells Tissues Organs*, **2004**, *177*, 132-138) implicated the TLR3 pathway in siRNA induction of innate immunity. Kariko *et al.* (*Cells Tissues Organs*, **2004**, *177*, 132-138) showed that TLR3 activation was concentration dependent, but the requirements for optimal TLR3 activation by dsRNA are presently unknown.

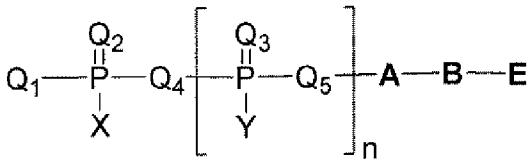
Activation of the innate immune response is advantageous in diseases ranging from viral infections to cancer. Activation of innate immunity by dsRNA and CpG DNA promoted antitumor effects in a mouse model (Whitmore *et al.*, *Cancer Res.*, **2004**, *64*, 5850-5860). iRNA agents should be potent adjuvants for vaccination against a variety of bacterial and viral pathogens. Such adjuvants should activate innate immunity, which, in turn should shape the adaptive immune response. Some iRNA sequences and modifications may better activate the innate immune response than others. This activation could come via any of the Toll-like receptor pathways that are known to bind nucleic acids: TLR3, the receptor for double-stranded RNA; TLR8, the receptor for single-stranded RNA; TLR9, the receptor with a preference for unmethylated CpG DNA; or PKR, the protein kinase activated by dsRNA. Each of these proteins is known to bind to molecules similar to the iRNA agents described.

There are a number of reasons that suppression of the innate immune system might be advantageous. As stimulation of the innate immune system results in inflammation, depression of this system when it is non-productive may benefit patients with asthma, serious local or systemic infections, or chronic inflammatory diseases such as inflammatory bowel disease, chronic obstructive pulmonary disease, and arthritis. Compounds that suppress the immune system may also be useful in treatment of cancer. In particular, disruption of the PKR pathway, which leads to activation of NF-κB, is attractive. NF-κB leads to inhibition of apoptosis that would otherwise eliminate defective cells (Pikarsky *et al.*, *Nature*, **2004**, *431*, 461-466) and also promotes metastatic growth (Huber *et al.*, *J. Clin. Invest.*, **2004**, *114*, 569-581).

SUMMARY

The present invention provides a novel class of iRNA agents that are capable of inducing an anti-viral or an antibacterial response, in particular, the induction of type I IFN, IL-18 and/or IL-1β by modulating RIG-I.

In one embodiment, the iRNA agents of the present invention are represented by formula I, as illustrated below:



(I)

or pharmaceutically acceptable salts or prodrugs thereof, wherein

Q_2 and Q_3 are each, independently NH, O or S;

X and Y are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

Q_4 and Q_5 are each independently O, CH_2, CHMe, CMe_2, CHF, CF_2, NH, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

n is 0, 1, 2, 3, 4 or 5; wherein each repeating unit can be the same or different;

A is absent or selected from the group consisting of single stranded oligonucleotide and double stranded oligonucleotide, each of which may be chemically modified;

B is absent or a linker/spacer;

E is a single stranded oligonucleotide or a double stranded oligonucleotide, each of which may be chemically modified or conjugated with a ligand;

with a proviso that when A and B are both absent and n is 0, 1 or 3, then Q_1, Q_2, Q_3, Q_4, Q_5, X and Y cannot be all oxygen.

In another embodiment of the present invention there are disclosed pharmaceutical compositions comprising a therapeutically effective amount of a iRNA agent of the invention in combination with a pharmaceutically acceptable carrier or excipient. In yet another embodiment of the invention are methods of treating diseases such as viral infections, bacterial infections, parasitic infections, tumors, allergies, autoimmune diseases, immunodeficiencies and immunosuppression. with said pharmaceutical compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

FIG. 1 is an ion-exchange HPLC analysis of purified RNA-Triphosphate

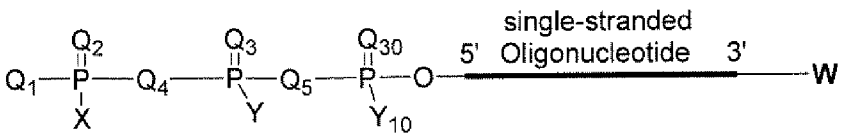
FIG. 2 is an LC-MS analysis of RNA 5'-Triphosphate

DETAILED DESCRIPTION

In a first embodiment of the compounds of the present invention are compounds represented by formula I as illustrated above, or a pharmaceutically acceptable salt, ester or prodrug thereof.

In one embodiment of the iRNA agents of the present invention are agents

represented by formula II as illustrated below, or a pharmaceutically acceptable salt or prodrug thereof:



Q_2 , Q_3 and Q_{30} are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

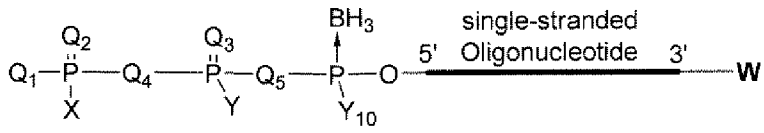
Q_4 and Q_5 are each independently O, CH_2, CHMe, CMe_2, CHF, CF_2, NH, NR_1 or S;

Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

W is H, OH or -G-L; where G is selected from the group consisting of –CONH-, -NHCO- , -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S- , -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand;

provided that when W is OH, then Q_1, Q_2, Q_3, Q_4, Q_5, Q_{30}, X, Y and Y_{10} cannot all be oxygen.

In one embodiment of the iRNA agents of the present invention are agents represented by formula III as illustrated below, or a pharmaceutically acceptable salt or prodrug thereof:



(III)

Q_2 and Q_3 are each, independently NH, O or S;

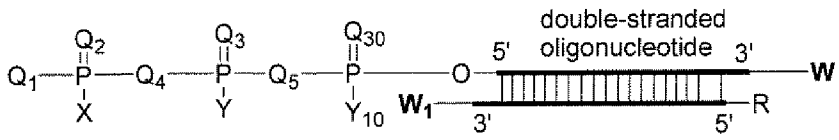
X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

Q_4 and Q_5 are each independently O, CH_2, CHMe, CMe_2, CHF, CF_2, NH, NR_1 or S;

Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

W is each independently H, OH or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand.

In one embodiment of the iRNA agents of the present invention are agents represented by formula IV as illustrated below, or a pharmaceutically acceptable salt or prodrug thereof:



(IV)

Q_2 , Q_3 and Q_{30} are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally

substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

Q_4 and Q_5 are each independently O, CH_2, CHMe, CMe_2, CHF, CF_2, NR^1, or S;

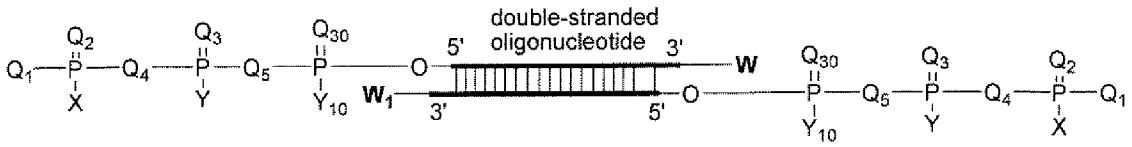
Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

R is H, phosphate or phosphorothioate;

W and W_1 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand;

provided that when W is OH, then Q_1, Q_2, Q_3, Q_4, Q_5, Q_{30}, X, Y and Y_{10} cannot be all oxygen.

In one embodiment of the iRNA agents of the present invention are agents represented by formula IVa as illustrated below, or a pharmaceutically acceptable salt or prodrug thereof:



(IVa)

Q_2 , Q_3 and Q_{30} are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl,

cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

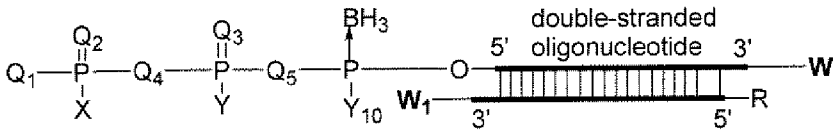
Q_4 and Q_5 are each independently O, CH_2, CHMe, CMe_2, CHF, CF_2, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

W and W_1 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand;

provided that when W is OH, then Q_1, Q_2, Q_3, Q_4, Q_5, Q_{30}, X, Y and Y_{10} cannot be all oxygen.

In one embodiment of the iRNA agents of the present invention are agents represented by formula V as illustrated below, or a pharmaceutically acceptable salt or prodrug thereof:



(V)

Q_2 and Q_3 are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

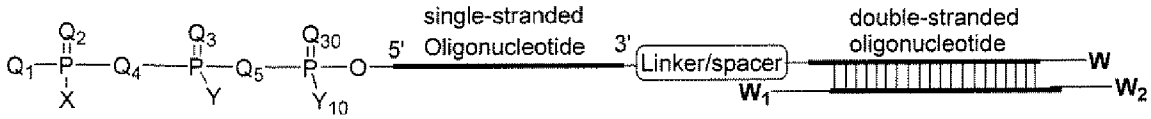
Q_4 and Q_5 are each independently O, CH_2, CHMe, CMe_2, CHF, CF_2, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

R is H, phosphate or phosphorothioate;

W and W_1 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand.

In one embodiment of the iRNA agents of the present invention are agents represented by formula VI as illustrated below, or a pharmaceutically acceptable salt or prodrug thereof:



(VI)

Q_2 , Q_3 and Q_{30} are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

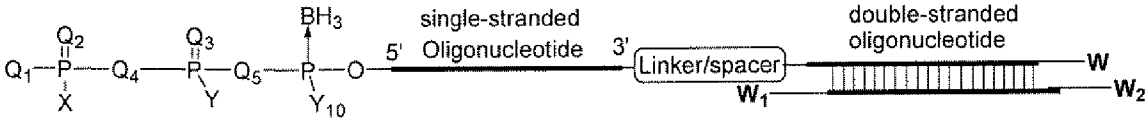
Q_4 and Q_5 are each independently O, CH_2, CHMe, CMe_2, CHF, CF_2, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

W, W_1 and W_2 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand;

Linker/spacer is selected from the group consisiting of phosphate, phosphorothioate, phosphorodithioate, alkylphosphonate, amide, ester, disulfide, thioether, oxime and hydrazone, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl and heteroaryl.

In one embodiment of the iRNA agents of the present invention are agents represented by formula VII as illustrated below, or a pharmaceutically acceptable salt or prodrug thereof:



(VII)

Q_2 and Q_3 are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, where R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted, and R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted,or OH;

Q_4 and Q_5 are each independently O, CH_2, CHF, CF_2, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, SR^1;

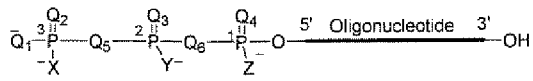
W, W_1 and W_2 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand;

Linker/spacer is selected from the group consisting of is selected from the group consisiting of phosphate, phosphorothioate, phosphorodithioate, alkylphosphonate,

amide, ester, disulfide, thioether, oxime and hydrazone, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl and heteroaryl.

Further representative sub species of the present invention are iRNA agents (1)-(15) of the formula (A), where Q_1, Q_2, Q_3, Q_4, Q_5, Q_6, X, Y and Z are delineated in Table 1:

TABLE 1

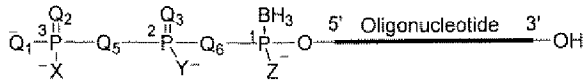


(A)

	X	Y	Z	Q1	Q2	Q3	Q4	Q5	Q6
1	O	O	S	O	O	O	O	O	O
2	O	S	S	O	O	O	O	O	O
3	S	O	S	O	O	O	O	O	O
4	S	S	O	O	O	O	O	O	O
5	S	S	S	O	O	O	O	O	O
6	O	O	S	O	O	O	S	O	O
7	O	O	S	S	O	O	O	O	O
8	S	O	S	S	O	O	O	O	O
9	S	O	S	S	O	O	S	O	O
10	O	O	S	S	O	O	S	O	O
11	O	O	O	S	O	O	O	O	O
12	O	O	O	S	S	O	O	O	O
13	O	O	O	S	O	S	O	O	O
14	S	O	O	S	O	O	S	O	O
15	O	O	O	S	O	O	S	O	O

Further representative sub species of the present invention are iRNA agents (16)-(31) of the formula (B), where Q_1, Q_2, Q_3, Q_5, Q_6, X, Y and Z are delineated in Table 2:

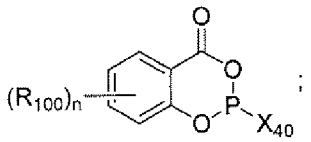
TABLE 2



(B)

	X	Y	Z	Q1	Q2	Q3	Q5	Q6
16	O	O	O	O	O	O	O	O
17	O	O	S	O	O	O	O	O
18	O	S	S	O	O	O	O	O
19	S	O	S	O	O	O	O	O
20	S	S	O	O	O	O	O	O
21	S	S	S	O	O	O	O	O
22	O	O	S	O	O	O	O	O
23	O	O	S	S	O	O	O	O
24	S	O	S	S	O	O	O	O
25	S	O	S	S	O	O	O	O
26	O	O	S	S	O	O	O	O
27	O	O	O	S	O	O	O	O
28	O	O	O	S	S	O	O	O
29	O	O	O	S	O	S	O	O
30	S	O	O	S	O	O	O	O
31	O	O	O	S	O	O	O	O

In one embodiment, oligonucleotide of the invention can be prepared by a A process of preparing nucleic acid molecule comprising a triphosphosphate or a triphosphate analog comprising the steps of: (a) protecting the 2'hydroxyl moiety with fluoride labile group or fluoride non-labile group; (b) converting the 5' hydroxyl moiety to triphosphate or triphosphate analog with a reagent selected from the group consisting of: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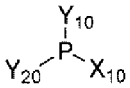
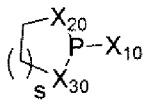
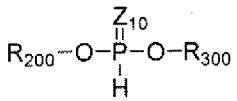
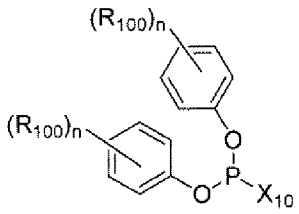

;

;


; and

;

wherein:
R_{100} is independently electron withdrawing group (EWG);
R_{200} and R_{300} are earch independly haloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, subsituted cycloalkyl, heterocycle, substituted heterocyclo;
Z_{10} is O, S, Se, BH_3 or NR';
X_{40} is Cl, dialkylamine or cyclic amine;
X_{10} is Cl, O-aryl or O-substituted aryl;
Y_{10} and Y_{20} are independently O-substituted alkyl; dialkylamine or cyclic amine, wherein the nitrogen is connected to the phosphorus;
X_{20} and X_{30} is independently O, CH_2, S, NR', wherein R' is H or aliphatic;
n is 1, 2, 3, 4, or 5; and
s is 0, 1, 2 or 3;

(c) synthesizing said nucleic acid molecule using a method selected from the group consisting of solid phase phosphoramidite, solution phase phosphoramidite, solid phase H-phosphonate, solution phase H-phosphonate, hybrid phase phosphoramidite, and hybrid phase H-phosphonate-based synthetic methods; and (d) removing the protecting group(s) and/or solid support. In one embodiment, the steps of the synthesis can be interchanged. For example, step (c) can precede step (a) and/or (b).

Suitable electron withdrawing groups include halogens, NO_2, CN, acyl, and sulfonyl. Suitable dialkylamines include $N(i\text{-}Pr)_2$.

In one embodiment, the sequence and motif of the oligonucleotide can be vary to induce RIG-1 activation in combination with 1) phosphorothioate linkage (5'-triphosphate with PS linkage – racemic at 5' and at internal positions; or 5'-triphosphate with PS linkage – optically pure (R and/or S configuration) at 5' and at internal positions); 2) chimeric oligonucleotides (5'-triphosphate-RNA-DNA-RNA-3' with CPG motif on the short DNA part to induce TLR activation. The DNA portion is 4-6 nucleotides in length, which exerts minimal effect on RNase H; 5'-Triphosphate-RNA-DNA-RNA-3' without CPG motif on the DNA part that has the potential to induce RNase H; 5'-triphosphate-RNA-DNA-RNA-3' with CPG motif on the DNA part that has the potential to induce RNase H and TLR activation; 5'-triphosphate-RNA-DNA-3' with CPG motif on the short DNA part to induce TLR activation where the DNA portion is 4-6 nucleotides in length, which exerts minimal effect on RNase H; 5'-triphosphate – RNA-DNA-3' without CPG motif on the DNA part that has the potential to induce RNase H; 5'-triphosphate – RNA-DNA-3' with CPG motif on the DNA part that has the potential to induce RNase H and TLR activation; 3) 5'-triphosphate oligoribonucleotide – insert sequence motifs known for immune stimulation; 4) place stimulating motif(s) – single motif or combinations there of described above – in the sense strand and preserve antisense strand for RNAi; and 5) place stimulating motif(s) – single motif or combinations there of described above – in the sense strand and introduce minimal chemical modification, that retain RNAi activity and that protect antisense strand from nucleases.

In one embodiment, the preferred oligonucleotide can have all natural 2'-deoxyribo and 2'-ribonuclesides, 2'-*O*-methyl (2'-OMe), 2'-*O*-methoxyethyl (2'-MOE), 2'-deoxy-2'-ribofluoro (2'-F), 2'-deoxy-2'-arabinofluoro (2'-araF) sugar modifications and combinations there of, with and without phosphorothioate backbone at the internucleoside linkages.

In one embodiment, the preferred nucleobase modifications includes 2-ThioU, 2'-amino-A, pseudouridine, inosine, 5-Me-U, 5-Me-C, chemically modified U analogues.

In one embodiment, the preferred Ligands includes PK modulators such as lipophiles, Cholesterol and analogs, bile acids, steroids, circulation enhancers – PEG with

different mol. wt. starting from 400 to up to 60,000 amu, small molecule protein binders (for e.g, naproxen or ibuprofen) and targeting ligands for receptor targeting, for e.g., folic acid, GalNAc and mannose.

Evaluation of the iRNA agent can include incubating the modified strand (with or without its complement, but preferably annealed to its complement) with a biological system, *e.g.*, a sample (*e.g*, a cell culture). The biological sample can be capable of expressing a component of the immune system. This allows identification of an iRNA agent that has an effect on the component. In one embodiment, the step of evaluating whether the iRNA agent modulates, *e.g*, stimulates or inhibits, an immune response includes evaluating expression of one or more growth factors, such as a cytokine or interleukin, or cell surface receptor protein, in a cell free, cell-based, or animal assay. Exemplary assay methods include ELISA and Western blot analysis. Growth factors that could be evaluated include TNFα, IL1α and β, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL11, IL12, IL13, IFNα and β, and IFNγ. In preferred embodiments, a test includes evaluating expression of one or more of the interleukins IL-18, IL-1β, IL-10, IL-12, and IL-6. Relevant cell surface receptors include the toll-like receptors, *e.g.*, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, or TLR9. In other preferred embodiments, a test includes evaluating expression of one or more of the toll-like receptors TL-3, TLR7, TLR8, or TLR9. Ligand interaction with TLR9 stimulates expression of NFκB. Therefore, testing whether an iRNA agent stimulates the immune response can include assaying for NFκB protein or mRNA expression.

In one embodiment, the step of testing whether the modified iRNA agent modulates, *e.g.*, stimulates, an immune response includes assaying for an interaction between the iRNA agent and a protein component of the immune system, *e.g.*, a growth factor, such as a cytokine or interleukin, or a cell surface receptor protein. For example, the test can include assaying for an interaction between the modified iRNA and a toll-like receptor, *e.g.*, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, or TLR9. In one preferred embodiment, testing includes assaying for an interaction with a toll-like receptor, *e.g.*, TLR-9. Exemplary assay methods include coimmunoprecipitation assays, bead-based co-isolation methods, nucleic acid footprint assays and colocalization experiments such as those facilitated by immunocytochemistry techniques.

In one embodiment, the candidate iRNA agent has the ability to sequence-specifically inhibit expression of a particular gene through the RNA interference pathway. For example, the iRNA agent can target a sequence that encodes a protein component of the immune system, such as a cytokine, cytokine receptor or Toll-like receptor (*e.g.*, TLR 3, TLR 7, TLR 8 and TLR 9,). The ability of an iRNA agent composition of the invention to inhibit gene expression can be measured using a variety of techniques including Northern blot analysis or RT-PCR for the specific mRNA or Western blot analysis for the amount of target protein present. In still another embodiment, a phenotype influenced by the amount of the protein can be detected.

In one embodiment, the candidate iRNA agent interacts with a protein component of the immune system, or has been modified not to interact, and also targets a sequence that is not involved in the immune system.

In one embodiment, the candidate iRNA agent includes at least one ribonucleotide modification (*e.g.*, as described below), and the presence of the modification modulates an immunostimulatory response (*e.g.*, as compared to what could be seen with an iRNA agent lacking the modification) when the modified iRNA agent is contacted with a cell or administered to a subject. In preferred embodiments, one or more natural bases of an iRNA agent are replaced by modified bases. In more preferred embodiments, one or more natural bases of an iRNA agent are replaced by modified bases. Particular modifications to the iRNA agent may prevent the strands of the agent from separating and subsequently interacting with the one or more protein components of the immune system. Particular modifications may also inhibit the interaction of a double stranded or single stranded iRNA agent from interacting with a protein component of the immune system to an extent that an immune system response is effectively prevented.

Chemical modifications can include modifications to the nucleotide base, the sugar, or the backbone. In one embodiment, the iRNA agent includes a substitution of an adenine with a 2-substituted purine (*e.g.*, 2-amino-adenine,), a 6-substituted purine, a 7-deaza-alkyl-substituted purine, a 7-deaza-alkenyl-substituted purine, a 7-deaza-alkynyl-substituted purine, or a purine that is not adenine (*e.g.*, guanine or inosine). In another embodiment, the candidate iRNA agent includes a substitution of a guanine with an inosine, an aminopurine, a 2-substituted guanine, a 7-deaza-alkyl-substituted guanine, a

7-deaza-alkenyl-substituted guanine, a 7-deaza-alkynyl-substituted guanine, or an O-6-alkylated guanine. In another embodiment, the candidate iRNA agent includes a substitution of a cytosine with a 5-substituted cytosine (*e.g.*, a 5-methyl cytosine), an N-4 substituted cytosine, a G-clamp, an analog of a G-clamp, a 2-thio-cytosine, a 4-thio-cytosine, or a uracil. In one embodiment, the candidate iRNA agent includes a substitution of a uracil with a 5-substituted uracil, a 4-thio-uracil, a 5-methyl-2-thio-uracil, a pseudouridine, a 1-alkylpseudouridine, a 3-alkylpseudouridine or a 2-thio-uracil. In one embodiment, the iRNA agent includes a 2′-deoxyfluoro, 2'-*O*-methyl, 2'-*O*-methoxyethyl, 2'-*O*-alkyl, 2'-*O*-alkoxyalkyl, 2'-*O*-allyl, 2'-*O*-propyl, 2'-*O*-(N-methyl-acetamide (NMA), 2'-*O*-(N,N-dimethylaminooxyethyl), or G-clamp modification. In one embodiment, the iRNA agent includes an arabinose-containing nucleoside that replaces a ribonucleoside. In another embodiment, the arabinose-containing nucleoside can be a 2'-fluoroarabinose-containing nucleoside, or a 2'-*O*-methylarabinose-containing nucleoside. In another embodiment, the iRNA agent includes a deoxynucleoside that replaces a ribonucleoside. In one embodiment, the deoxynucleoside is a 2'-fluorodeoxynucleoside, or a 2'-*O*-methyldeoxynucleoside.

In one embodiment, an immunoselective iRNA agent includes at least one backbone modification, *e.g.*, a phosphorothioate, boronaphosphate, methylphosphonate or dithioate modification. In another embodiment, the iRNA agent includes a P-alkyl modification in the linkages between one or more of the terminal nucleotides of an iRNA agent. In another embodiment, the sense and/or antisense strand is substantially free of stereogenic phosphorus atoms having an Rp configuration, and in another embodiment, the sense and/or antisense strand is substantially free of stereogenic phosphorus atoms having an Sp configuration.

In another embodiment, one or more terminal nucleotides of an iRNA agent include a sugar modification, *e.g.*, a 2' or 3' sugar modification. In one embodiment, the iRNA agent includes at least two sugar 2' modifications. Exemplary sugar modifications include, for example, a 2'-fluoro nucleotide, a 2'-*O*-alkyl nucleotide, a 2'-*O*-alkoxyalkyl nucleotide, a 2'-*O*-allyl nucleotide, a 2' *O*-propyl nucleotide, a 2'-*O*-methylated nucleotide (2'-*O*-Me), a 2'-deoxy nucleotide, a 2′-deoxyfluoro nucleotide, a 2'-*O*-methoxyethyl nucleotide (2'-*O*-MOE), a 2'-*O*-N-MeAcetamide nucleotide (2'-*O*-

NMA), a 2'-*O*-dimethylaminoethyloxyethyl nucleotide (2'-*O*- DMAEOE), a 2'-aminopropyl, a 2'-hydroxy, a 2'-ara-fluoro, or 3'-amidate (3'-NH in place of 3'-O), a locked nucleic acid (LNA), extended ethylene nucleic acid (ENA), hexose nucleic acid (HNA), or cyclohexene nucleic acid (CeNA).

In one embodiment, the iRNA agent includes a 3' sugar modification, *e.g.*, a 3'-*O*-Me modification. Preferably a 3'-*O*-Me modification is on the sense strand of the iRNA agent.

In some embodiments, the iRNA agent includes a 5'-alkyl-pyrimidine *e.g* 5'-methyl-pyrimidine (*e.g.*, a 5'-methyl-uridine modification or a 5'-methyl-cytodine modification).

The modifications described herein can be combined onto a single candidate iRNA agent. For example, in one embodiment, at least one nucleotide of an iRNA agent has a phosphorothioate linkage and at least one nucleotide has a 2' sugar modification, *e.g.*, a 2'-*O*-Me or 2'-deoxyfluoro modification. In another embodiment, at least one nucleotide of a candidate iRNA agent has a 5'-Me-pyrimidine and a 2' sugar modification, *e.g.*, a 2'-deoxyfluoro or 2'-*O*-Me modification.

In one embodiment, the iRNA agent includes a nucleobase modification, such as a cationic modification, such as a 3'-abasic cationic modification. The cationic modification can be *e.g.*, an alkylamino-dT (*e.g.*, a C6 amino-dT), an allylamino conjugate, a pyrrolidine conjugate, a pthalamido, a porphyrin, or a hydroxyprolinol conjugate.

In one embodiment, the iRNA agent includes at least one ribonucleotide modification on the sense strand and at least one ribonucleotide modification on the antisense strand, and the ribonucleotide modifications on the two strands are different.

In another embodiment, the iRNA agent, *e.g.*, an iRNA agent that can inhibit an immune response, may include a conjugate on one or more nucleotides of the iRNA agent. The conjugate can be, for example, a lipophile, a terpene, a protein-binding agent, a vitamin, a carbohydrate, or a peptide. For example, the conjugate can be naproxen, nitroindole (or another conjugate that contributes to stacking interactions), folate, ibuprofen, or a C5 pyrimidine linker. In other embodiments, the conjugates are glyceride

lipid conjugates (*e.g.*, a dialkyl glyceride derivatives), vitamin E conjugates, or thio-cholesterols.

In one embodiment, the conjugate is cholesterol, and the cholesterol is conjugated to the sense strand of the iRNA agent, *e.g.*, by a pyrrolidine linker, serinol linker, or hydroxyprolinol linker. In other embodiments, the conjugate is a dU-cholesterol, or cholesterol is conjugated to the iRNA agent by a disulfide linkage. In another embodiment, the conjugate is cholanic acid, and the cholanic acid is attached to the sense strand or the antisense strand. In one embodiment, the cholanic acid is attached to the sense strand and the antisense.

In another embodiment, one or more nucleotides have a 2'-5' linkage, and preferably, the 2'-5' linkage is on the sense strand.

In one embodiment, the iRNA agent includes an L-sugar, preferably on the sense strand.

In one embodiment, the iRNA agent includes a methylphosphonate.

In one embodiment, the iRNA agent has been modified by replacing one or more ribonucleotides with deoxyribonucleotides. Preferably, adjacent deoxyribonucleotides are joined by phosphorothioate linkages, and the iRNA agent does not include more than four consecutive deoxyribonucleotides on the sense or the antisense strands.

In some embodiments, the iRNA agent includes a difluorotoluyl (DFT) modification, *e.g.*, 2,4-difluorotoluyl uracil, or a guanidine to inosine substitution.

In one embodiment, the iRNA agent includes a 5'-uridine-adenine-3' (5'-UA-3') dinucleotide wherein the uridine is a 2'-modified nucleotide, or a 5'-uridine-guanine-3' (5'-UG-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a 5'-cytidine-adenine-3' (5'-CA-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a 5'-uridine-uridine-3' (5'-UU-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a 5'-cytidine-cytidine-3' (5'-CC-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a 5'-cytidine-uridine-3' (5'-CU-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a 5'-uridine-cytidine-3' (5'-UC-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide. The chemically modified nucleotide in the iRNA agent may be a 2'-*O*-methylated nucleotide. In some embodiments, the modified nucleotide can be a 2'-deoxy nucleotide, a 2′-

deoxyfluoro nucleotide, a 2'-*O*-methoxyethyl nucleotide, a 2'-*O*-NMA, a 2'-DMAEOE, a 2'-aminopropyl, 2'-hydroxy, or a 2'-ara-fluoro, or 3'-amidate (3'-NH in place of 3'-O), a locked nucleic acid (LNA), extended nucleic acid (ENA), hexose nucleic acid (HNA), or cyclohexene nucleic acid (CeNA).

In one embodiment, the iRNA agent has a single overhang, *e.g.*, one end of the iRNA agent has a 3' or 5' overhang and the other end of the iRNA agent is a blunt end. In another embodiment, the iRNA agent has a double overhang, *e.g.*, both ends of the iRNA agent have a 3' or 5' overhang, such as a dinucleotide overhang. In another embodiment, both ends of the iRNA agent have blunt ends.

In one embodiment, the iRNA agent includes a sense RNA strand and an antisense RNA strand, and the antisense RNA strand is 18 - 30 nucleotides in length. In another embodiment, the iRNA agent includes a nucleotide overhang having 1 to 4 unpaired nucleotides, which may be at the 3'-end of the antisense RNA strand, and the nucleotide overhang may have the nucleotide sequence 5'-GC-3' or 5'-CGC-3'. The unpaired nucleotides may have at least one phosphorothioate dinucleotide linkage, and at least one of the unpaired nucleotides may be chemically modified in the 2'-position. In one embodiment, the double strand region of the candidate iRNA agent includes phosphorothioate linkages on one or both of the sense and antisense strands. In a preferred embodiment, the candidate iRNA agent includes phosphorothioate linkages between nucleotides 1 through 5 of the 5' or 3' end of the sense or antisense agent.

In one embodiment, the antisense RNA strand and the sense RNA strand are connected with a linker. The chemical linker may be a hexaethylene glycol linker, a poly-(oxyphosphinico-oxy-1,3-propandiol) linker, an allyl linker, or a polyethylene glycol linker. Use of a linker to connect the antisense and sense strands, will inhibit strand separation in vivo, thereby inhibiting immunostimulation.

In another embodiment, one or more modifications of the iRNA agent can increase the ratio of double-stranded to single-stranded iRNA agent in a biological system (*e.g.*, in the blood stream or in serum samples). Such modifications may decrease the dissociation constant (K_D) between the sense and antisense strands. In some embodiments, the modification that increases the ratio of double-stranded to single-stranded iRNA agent in a biological system is a chemical linker, such as a hexaethylene

glycol linker, poly-(oxyphosphinico-oxy-1,3-propandiol) linker, allyl linker, or polyethylene glycol linker that binds the two strands together. In one embodiment, the linker includes an ester, and the linker can be cleaved by an esterase. In some embodiments, the modification is a high affinity chemical modification, such as 2'-F, LNA, ENA, 2'-*O*-MOE, and C-5-propynyl pyrimidines or G-clamp and its analogs. In yet other embodiments, the modification is a chemical crosslink, *e.g.*, a disulfide containing crosslink.

In another embodiment, the immunoselective iRNA agent can include at least two modifications. The modifications can differ from one another, and may be applied to different RNA strands of a double-stranded iRNA agent. For example, the sense strand can include at least one modification, and the antisense strand can include a modification that differs from the modification or modifications on the sense strand. In another example, the sense strand can include at least two different modifications, and the antisense strand can include at least one modification that differs from the two different modifications on the sense strand. Accordingly, the sense strand can include multiple different modifications, and the antisense strand can include further multiple modifications, some of which are the same or unique from the modifications on the sense strand.

In one aspect, the invention relates to methods of designing an iRNA agent that binds specifically to one of the protein components of the immune system and either inhibits or stimulates the immune cascade. The method may include designing an iRNA agent that includes a sense strand, and an antisense strand sufficiently complementary to hybridize to the sense strand, designing the strands such that one or more of the nucleotides on the sense and/or antisense strand are modified as described herein. The iRNA agent may be further synthesized and tested in an *in vitro* or *in vivo* system for binding to a protein component of the immune system, *e.g.*, as described above. For example, the iRNA agent can be tested by assaying for an interaction with a component of the immune system, *e.g.*, a growth factor, such as a cytokine or interleukin protein, or cell-surface receptor. The assay can include introducing an iRNA agent into a cell, maintaining the cell under conditions suitable for expressing a component of the immune system, and determining whether the iRNA agent can interact with the component of the

immune system, *e.g.*, by co-immunoprecipitation experiments, or colocalization experiments, such as those facilitated by immunocytochemistry techniques. The cell may be a mammalian cell. The iRNA agent may be tested for an immune stimulatory or immune inhibitory effect in a cellular assay. The iRNA agent may be tested in an *in vivo* system by administering the iRNA agent to a mammal, such as a mouse, then examining the spleen for enlargement or increased cell proliferation, or for an increase in production of, for example, one or more interleukin proteins, such as IL10, IL12 and/or IL6.

In some embodiments, the candidate iRNA agent is tested in a first system, *e.g.*, a cell-free system or cell-based system, and then retested. The retest can be in the same or different assays. For example, the same or a different cell-based or cell-free assay can be used to confirm activity, or an animal-based system can be used to confirm activity (*e.g.*, gene-silencing activity, or stimulation or inhibition of the immune system) or lack of activity with respect to its effect on an immune response. In some embodiments, the candidate iRNA agent is tested first in a cell-free or cell-based system and is then retested in an animal-based system.

In one aspect, the invention features an iRNA agent that includes at least one ribonucleotide modification, *e.g.*, a ribonucleotide agent described above, and the presence of the modification inhibits or stimulates an immunostimulatory response (*e.g.*, as compared to what could be seen with an iRNA agent lacking the modification) when the modified iRNA agent is contacted with a cell or administered to a subject, *e.g.*, a mammalian subject, such as a human. In preferred embodiments, one or more natural bases of an iRNA agent are replaced by modified bases.

In one aspect the invention features a method of evaluating an iRNA agent that includes providing a candidate single stranded iRNA agent having at least one ribonucleotide modification; contacting the candidate single stranded iRNA agent to a cell-free system, cell, or animal; and evaluating the immune response in the cell-free system, cell, or animal as compared to an immune response in a cell-free system, cell, or animal that is contacted with an unmodified single stranded iRNA agent. The candidate single stranded iRNA agent stimulates an immune response to a lesser or greater extent than a reference. For example, an unmodified iRNA agent is determined to be an iRNA agent that modulates an immune system response. In one embodiment, the candidate

single-stranded iRNA agent is 15-2000 nucleotides in length (*e.g.*, 17, 19, 21, 23, 25, 27, 28, 29, 30, 100, 500, 1000, or 1500 nucleotides in length).

In one aspect, the invention relates to an iRNA agent with dual function: an iRNA agent that either inhibits or stimulates the immune system and also sequence-specifically targets an mRNA of therapeutic relevance for degradation via the RISC-mediated RNA interference pathway. The immunoselective iRNA agent (*e.g.*, an iRNA agent that inhibits or stimulates immunostimulation) is prepared by a process described herein, *e.g.*, a process that includes providing a first oligonucleotide that is sufficiently complementary to a target nucleic acid to anneal to the target and a second oligonucleotide sufficiently complementary to anneal to the first oligonucleotide. The first and/or second oligonucleotides include one or more modified nucleotides or nucleotide linkages described above. One or both of the antisense and sense strands, or preferably annealed antisense and sense strands, is tested, *e.g.*, as described herein, for the ability to modulate, *e.g.*, stimulate or inhibit an immune response, *e.g.*, when administered to a test subject. For example, if the iRNA agent does not stimulate an immune response to a preselected magnitude, *e.g.*, to the magnitude of an iRNA agent lacking the one or more modified nucleotides then the agent is determined to inhibit immunostimulation. The modified iRNA agent, *e.g.*, one determined to inhibit or stimulate immunostimulation, is further tested to determine if it can decrease expression of a target mRNA, *e.g.*, by the mechanism of RNA interference, *e.g.*, to the same extent as the unmodified version of the iRNA agent.

In another embodiment, the invention relates to the silencing of the gene for a particular component of the immune system, *e.g.* toll-like receptor 7 (TLR7) or toll-like receptor 9 (TLR9). In this embodiment, the iRNA agent is designed to be complementary to a region of the mRNA for the component and the iRNA agent decreases the expression of the target through the mechanism of RNA interference.

In still another aspect, the invention relates to a pharmaceutical composition including an immunoselective iRNA agent, as described above, and a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be an aqueous solution, such as phosphate buffered saline, or it may include a micellar structure, such as a liposome, capsid, capsoid, polymeric nanocapsule, or polymeric microcapsule.

In yet a further aspect, the invention relates to a method for treating a disease or disorder in a subject. The method includes identifying a subject having or at risk for developing the disease, administering a pharmaceutical composition containing an immunoselective iRNA agent having one or more of the modified nucleotides or linkages described above, and a pharmaceutically acceptable carrier. The subject may be monitored for an effect on the immune system, *e.g.*, an immunostimulatory or immunoinhibitory response, such as by monitoring for increased expression of a growth factor, such as a cytokine or a cell-surface receptor (*e.g.*, a Toll-like receptor) as described above. Cytokines of interest can be those expressed from T cells, B cells, monocytes, macrophages, dendritic cells, or natural killer cells of the subject. The assays can be performed using blood or serum samples from the subject. The disease or disorder can be one where it is particularly undesirable to stimulate the immune system, *e.g.*, in a patient that has received organ, tissue or bone marrow transplants. In another alternative, the disease or disorder can be one where it is particularly desirable to stimulate the immune system, *e.g.*, in patients with cancer or viral diseases. In one embodiment, the subject is immunocompromised, and an iRNA agent that includes nucleotide modifications stimulates an immune response in a cell to a greater extent than an iRNA agent that does not include nucleotide modifications. The subject may be a mammal, such as a human.

In a preferred embodiment, administration of an immunoselective iRNA agent is for treatment of a disease or disorder present in the subject. In another preferred embodiment, administration of the iRNA agent is for prophylactic treatment.

It is therefore an object of the present invention to provide polynucleotides/oligonucleotides which are capable of stimulating an anti-viral response, in particular, a type I IFN response. It is another object of the present invention to provide a pharmaceutical composition capable of inducing an anti-viral response, in particular, type I IFN production, in a patient for the prevention and treatment of diseases and disorders such as viral infection. It is also an object of the present invention to provide a pharmaceutical compostion for treating tumor.

The disease and/or disorder include, but are not limited to infections, tumor, allergy, multiple sclerosis, and immune disorders.

Infections include, but are not limited to, viral infections, bacterial infections, anthrax, parasitic infections, fungal infections and prion infection.

Viral infections include, but are not limited to, infection by hepatitis C, hepatitis B, herpes simplex virus (HSV), HIV-AIDS, poliovirus, encephalomyocarditis virus (EMCV) and smallpox virus. Examples of (+) strand RNA viruses which can be targeted for inhibition include, without limitation, picornaviruses, caliciviruses, nodaviruses, coronaviruses, arteriviruses, flaviviruses, and togaviruses. Examples of picornaviruses include enterovirus (poliovirus 1), rhinovirus (human rhinovirus 1A), hepatovirus (hepatitis A virus), cardiovirus (encephalomyocarditis virus), aphthovirus (foot-and-mouth disease virus O), and parechovirus (human echovirus 22). Examples of caliciviruses include vesiculovirus (swine vesicular exanthema virus), lagovirus (rabbit hemorrhagic disease virus), "Norwalk-like viruses" (Norwalk virus), "Sapporo-like viruses" (Sapporo virus), and "hepatitis E-like viruses" (hepatitis E virus). Betanodavirus (striped jack nervous necrosis virus) is the representative nodavirus. Coronaviruses include coronavirus (avian infections bronchitis virus) and torovirus (Berne virus). Arterivirus (equine arteritis virus) is the representative arteriviridus. Togavirises include alphavirus (Sindbis virus) and rubivirus (Rubella virus). Finally, the flaviviruses include flavivirus (Yellow fever virus), pestivirus (bovine diarrhea virus), and hepacivirus (hepatitis C virus).

In certain embodiments, the viral infections are selected from chronic hepatitis B, chronic hepatitis C, HIV infection, RSV infection, HSV infection, VSV infection, CMV infection, and influenza infection.

In one embodiment, the infection to be prevented and/or treated is upper respiratory tract infections caused by viruses and/or bacteria. In another embodiment, the infection to be prevented and/or treated is bird flu.

Bacterial infections include, but are not limited to, streptococci, staphylococci, E. coli, pseudomonas.

In one embodiment, bacterial infection is intracellular bacterial infection. Intracellular bacterial infection refers to infection by intracellular bacteria such as mycobacteria (tuberculosis), chlamydia, mycoplasma, listeria, and facultative intracellular bacteria such as staphylococcus aureus.

Parasitic infections include, but are not limited to, worm infections, in particular, intestinal worm infection.

Tumors include both benign and malignant tumors (i.e., cancer).

Cancers include, but are not limited to biliary tract cancer, brain cancer, breast cancer, cervical cancer, choriocarcinoma, colon cancer, endometrial cancer, esophageal cancer, gastric cancer, intraepithelial neoplasm, leukemia, lymphoma, liver cancer, lung cancer, melanoma, myelomas, neuroblastoma, oral cancer, ovarian cancer, pancreatic cancer, prostate cancer, rectal cancer, sarcoma, skin cancer, testicular cancer, thyroid cancer and renal cancer.

In certain embodiments, cancers are selected from hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell leukemia, chronic myeloid leukemia, non-Hodgkin's lymphoma, multiple myeloma, follicular lymphoma, malignant melanoma, squamous cell carcinoma, renal cell carcinoma, prostate carcinoma, bladder cell carcinoma, breast carcinoma, ovarian carcinoma, non-small cell lung cancer, small cell lung cancer, hepatocellular carcinoma, basaliom, colon carcinoma, cervical dysplasia, and Kaposi's sarcoma (AIDS-related and non-AIDS related).

Allergies include, but are not limited to, respiratory allergies, contact allergies and food allergies.

Immune disorders include, but are not limited to, autoimmune diseases, immunodeficiency, and immunosuppression.

Autoimmune diseases include, but are not limited to, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosis, automimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing, loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis,

Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves' disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis. Immunodeficiencies include, but are not limited to, spontaneous immunodeficiency, acquired immunodeficiency (including AIDS), drug-induced immunodeficiency (such as that induced by immunosuppressants used in transplantation and chemotherapeutic agents used for treating cancer), immunosuppression caused by chronic hemodialysis, trauma or surgical procedures.

Immunosuppression includes, but is not limited to, bone marrow suppression by cytotoxic chemotherapy.

In one embodiment, the pharmaceutical composition is a tumor vaccine. The iRNA agent described in the invention or the bacterial RNA may induce tumor cell apoptosis through binding to RIG-I, induce type I IFN_1 IL-18 and/or IL-1β production by the tumor cells, directly and/or indirectly activate effector cells of innate immunity such as NK cells, NKT cells, and γδ T cells, and/or directly and/or indirectly inactivate suppressor T cells, thereby leading to tumor cell growth inhibition and/or destruction.

Tumor cells which have been stimulated with an RIG-I ligand, such as the iRNA agent described in the present invention or a bacterial RNA, may also be used as a tumor vaccine.

In a preferred embodiment, the RNA oligonucleotide is a single-stranded RNA oligonucleotide which does not contain any sequence which is capable of forming any intramolecular or intermolecular double-stranded structure with itself under physiological condition, in particular, physiological condition inside a cell, and the nucleotide sequence of the ssRNA is complentary to a disease/disorder-related RNA.

In one embodiment, the disease/disorder-related RNA is an mRNA of a disease/disorder- related gene. In another embodiment, the disease/disorder-related RNA is a miRNA. The disease/disorder-related RNA may be a endogenous cellular RNA, a viral RNA, a RNA from an invading microorganism or organism such as a bacterium, a fungus, or a parasite.

The degree of complementarity is preferably at least 50%, 60%, 70%, more preferably at least 75%, 80%, 85%, 90%, even more preferably at least 95%, 96%, 97%, 98%, 99%, and most preferably 100%.

In one aspect, the invention features a method of increasing the ratio of double stranded iRNA (dsiRNA) agent to single stranded iRNA (ssiRNA) agent in a human by administering to the human a dsiRNA agent that includes one or more modifications that inhibit disassociation of the dsiRNA agent as compared to a dsiRNA agent that does not include the modifications. In one embodiment, the modifications include a chemical linker, such as a hexaethylene glycol linker, poly(oxyphosphinico-oxy-1,3-propandiol) linker, allyl linker, or polyethylene glycol linker. In another embodiment, the modifications increase the melting temperature of the dsiRNA as compared to a dsiRNA that does not include the modifications. Such modifications can include a locked nucleic acid, G-clamp, 2'-O-methyl, 2'-fluoro, 2'-*O*-methoxyethyl, 2-thio-pyrimidine, 2-amino-adenine or pseudouridine. In some embodiments, the modifications of the dsiRNA agent occur only in the sense strand of the dsiRNA agent or only in the antisense strand of the dsiRNA agent.

In one aspect, the invention features a method of selecting a patient suitable for treatment with an immunoselective iRNA agent described herein. In one embodiement, the selection of the patient is based on need for either immunostimulation or immunosuppression. In another embodiment, selection is based on the identification of a patient in need of decreased expression of a gene not involved in the immune system, and also in need of increased or decreased immune system function. The patient's need with respect to immune system function will determine which modifications will be incorporated into the therapeutic immunoselective iRNA agent.

In one aspect, the invention features an immunoselective iRNA agent that stimulates the immune system. For example, a stimulatory immunoselective iRNA agent includes one or more modifications, *e.g.*, modifications described herein for stimulating the immune system. For example, the modified iRNA agent will stimulate the immune system to a greater extent than an iRNA agent that does not include the one or more modifications. These stimulatory immunoselective iRNA agents can be administered to immunocompromised patients, or can be administered with a second therapy to off-set immunocompromising effects of the second therapy. The second therapeutic can be a chemotherapy or antibiotic, for example. A stimulatory immunoselective iRNA agent can be administered to a subject having or at risk for developing a tumor, an autoimmune

disease, airway inflammation (*e.g.*, asthma), an allergy (*e.g.*, a food or respiratory allergy), or a pathogenic disease, such as a disease caused by a bacteria, fungus, parasite, virus or viroid particle, or prion.

Definitions

The term "linker" or "spacer" generally refers to any moiety that can be attached to an oligoribonucleotide by way of covalent or non-covalent bonding through a sugar, a base, or the backbone. The linker/spacer can be used to attach two or more nucleosides or can be attached to the 5' and/or 3' terminal nucleotide in the oligoribonucleotide. Such linker can be either a non-nucleotidic linker or a nucleotidic linker.

The term "non-nucleotidic linker" generally refers to a chemical moiety other than a nucleotidic linkage that can be attached to an oligoribonucleotide by way of covalent or non-covalent bonding. Preferably such non-nucleotidic linker is from about 2 angstroms to about 200 angstroms in length, and may be either in a cis or trans orientation, (e.g. $d(T)_n$; wherein n is 1-10) or non-nucleotidic (for example a linker described herein, e.g. optionally substituted alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl or heteroaryl).

The term "nucleotidic linkage" generally refers to a chemical linkage to join two nucleosides through their sugars (e.g. 3'-3', 2'-3',2'-5', 3'-5') consisting of a phosphate, non-phosphate, charged, or neutral group (e.g., phosphodiester, phosphorothioate, phosphorodithioate, alkylphosphonate (e.g. methylphosphonate), amide, ester, disulfide, thioether, oxime and hydrazone linkage between adjacent nucleosides.

In one embodiment, the linker/spacer between the two oligonucleotides comprises a cleavable linking group, for example a group that is potentially biodegradable by enzymes present in the organism such as nucleases and proteases or cleavable at acidic pH or under reductive conditions, such as by glutathione present at high levels intracelullarly. Some exemplary cleavable linking groups include, but are not limited to, disulfides, amides, esters, peptide linkages and phosphodiesters. Copending United

States Application Serial No. 10/985,426, filed November 9, 2004, describes cleavable tethers that are amenable for use as spacers comprising cleavable groups.

The cleavable linking group can be internal to the spacer or may be present at one or both terminal ends of the spacer. In one embodiment, the cleavable linking group is between one of the oligonucleotides and the spacer. In one embodiment, the cleavable linking group is present on both ends of the spacer. In one embodiment, the cleavable linking group is internal to the spacer.

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

The term "aliphatic" refers to non-aromatic moiety that may contain any combination of carbon atoms, hydrogen atoms, halogen atoms, oxygen, nitrogen or other atoms, and optionally contain one or more units of unsaturation, e.g., double and/or triple bonds. An aliphatic group may be straight chained, branched or cyclic and preferably contains between about 1 and about 24 carbon atoms, more typically between about 1 and about 12 carbon atoms. In addition to aliphatic hydrocarbon groups, aliphatic groups include, for example, polyalkoxyalkyls, such as polyalkylene glycols, polyamines, and polyimines, for example. Such aliphatic groups may be further substituted.

The term "alkyl" refers to a hydrocarbon chain that may be a straight chain or branched chain, containing the indicated number of carbon atoms. For example, C_1-C_{12} alkyl indicates that the group may have from 1 to 12 (inclusive) carbon atoms in it. The term "haloalkyl" refers to an alkyl in which one or more hydrogen atoms are replaced by halo, and includes alkyl moieties in which all hydrogens have been replaced by halo (*e.g.*, perfluoroalkyl). Alkyl and haloalkyl groups may be optionally inserted with O, N, or S. The terms "aralkyl" refers to an alkyl moiety in which an alkyl hydrogen atom is replaced by an aryl group. Aralkyl includes groups in which more than one hydrogen atom has been replaced by an aryl group. Examples of "aralkyl" include benzyl, 9-fluorenyl, benzhydryl, and trityl groups.

The term "alkenyl" refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more double bonds. Examples of a typical alkenyl include, but not limited to, allyl, propenyl, 2-butenyl, 3-hexenyl and 3-

octenyl groups. The term "alkynyl" refers to a straight or branched hydrocarbon chain containing 2-8 carbon atoms and characterized in having one or more triple bonds. Some examples of a typical alkynyl are ethynyl, 2-propynyl, and 3-methylbutynyl, and propargyl. The sp^2 and sp^3 carbons may optionally serve as the point of attachment of the alkenyl and alkynyl groups, respectively.

The terms "alkylamino" and "dialkylamino" refer to –NH(alkyl) and –N $(alkyl)_2$ radicals respectively. The term "aralkylamino" refers to a –NH(aralkyl) radical. The term "alkoxy" refers to an -O-alkyl radical, and the terms "cycloalkoxy" and "aralkoxy" refer to an -O-cycloalkyl and O-aralkyl radicals respectively. The term "siloxy" refers to a R_3SiO- radical. The term "mercapto" refers to an SH radical. The term "thioalkoxy" refers to an -S-alkyl radical.

The term "alkylene" refers to a divalent alkyl (*i.e.*, -R-), *e.g.*, $-CH_2-$, $-CH_2CH_2-$, and $-CH_2CH_2CH_2-$. The term "alkylenedioxo" refers to a divalent species of the structure -O-R-O-, in which R represents an alkylene.

The term "aryl" refers to an aromatic monocyclic, bicyclic, or tricyclic hydrocarbon ring system, wherein any ring atom can be substituted. Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, anthracenyl, and pyrenyl.

The term "cycloalkyl" as employed herein includes saturated cyclic, bicyclic, tricyclic,or polycyclic hydrocarbon groups having 3 to 12 carbons, wherein any ring atom can be substituted. The cycloalkyl groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond or a common carbon atom (*e.g.*, spiro-fused rings). Examples of cycloalkyl moieties include, but are not limited to, cyclohexyl, adamantyl, and norbornyl.

The term "heterocyclyl" refers to a nonaromatic 3-10 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom can be substituted. The heterocyclyl groups herein described may also contain fused rings. Fused rings are rings that share a common carbon-carbon bond or a common carbon atom (*e.g.*, spiro-fused rings). Examples of heterocyclyl include, but are not

limited to tetrahydrofuranyl, tetrahydropyranyl, piperidinyl, morpholino, pyrrolinyl and pyrrolidinyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein any ring atom can be substituted. The heteroaryl groups herein described may also contain fused rings that share a common carbon-carbon bond.

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

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

The term "substituents" refers to a group "substituted" on an alkyl, cycloalkyl, alkenyl, alkynyl, heterocyclyl, heterocycloalkenyl, cycloalkenyl, aryl, or heteroaryl group at any atom of that group. Suitable substituents include, without limitation, alkyl, alkenyl, alkynyl, alkoxy, halo, hydroxy, cyano, nitro, amino, SO_3H, sulfate, phosphate, perfluoroalkyl, perfluoroalkoxy, methylenedioxy, ethylenedioxy, carboxyl, oxo, thioxo, imino (alkyl, aryl, aralkyl), $S(O)_n$alkyl (where n is 0-2), $S(O)_n$ aryl (where n is 0-2), $S(O)_n$ heteroaryl (where n is 0-2), $S(O)_n$ heterocyclyl (where n is 0-2), amine (mono-, di-, alkyl, cycloalkyl, aralkyl, heteroaralkyl, and combinations thereof), ester (alkyl, aralkyl, heteroaralkyl), amide (mono-, di-, alkyl, aralkyl, heteroaralkyl, and combinations thereof), sulfonamide (mono-, di-, alkyl, aralkyl, heteroaralkyl, and combinations thereof), unsubstituted aryl, unsubstituted heteroaryl, unsubstituted heterocyclyl, and unsubstituted cycloalkyl. In one aspect, the substituents on a group are independently any one single, or any subset of the aforementioned substituents.

A "protected" moiety refers to a reactive functional group, *e.g.*, a hydroxyl group or an amino group, or a class of molecules, *e.g.*, sugars, having one or more functional groups, in which the reactivity of the functional group is temporarily blocked by the

presence of an attached protecting group. Protecting groups useful for the monomers and methods described herein can be found, *e.g.*, in Greene, T.W., *Protective Groups in Organic Synthesis* (John Wiley and Sons: New York), 1981, which is hereby incorporated by reference.

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

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

Ligand

In the present invention, the ligand is an aromatic group, aralkyl group, or the radical of a steroid, bile acid, lipid, folic acid, pyridoxal, B12, riboflavin, biotin, polycyclic compound, crown ether, intercalator, cleaver molecule, protein-binding agent, carbohydrate, or an optionally substituted saturated 5-membered ring. In certain instances, the ligand is an aralkyl group, e.g., a 2-arylpropanoyl moiety. The structural features of the ligand are selected so that the ligand will bind to at least one protein in vivo. In certain embodiments, the structural features of the ligand are selected so that ligand binds to serum, vascular, or cellular proteins. In certain embodiments, the structural features of the ligand promote binding to albumin, an immunoglobulin, a lipoprotein, α-2-macroglubulin, or α-1-glycoprotein.

A large number of steroids are known in the art and are amenable to the present invention. Representative examples of steriods include cholesterol, 5β-cholanic acid,

progesterone, aldosterone, dehydroaldosterone, isoandrosterone, esterone, estradiol, ergosterol, dehydroergosterol, lanosterol, 4-cholesten-3-one, guggulsterone, testosterone, nortestosterone, formestane, hydroxyecdysone, ketoestriol, corticosterone, dienestrol, dihydroxypregnanone, pregnanone, copornmon, equilenin, equilin, estriol, ethinylestradiol, mestranol, moxestrol, mytatrienediol, quinestradiol, quinestrol, helvolic acid, protostadiene, fusidic acid, cycloartenol, tricallol, cucurbitanin cedrelone, euphol, dammerenediol, parkeol, dexametasone, methylprednisolone, prednisolone, hydrocortisone, parametasone, betametasone, cortisone, fluocinonide, fluorometholone, halcinonide, and budesonide, or any one of them further substituted with one or more of hydroxyl, halogen, amino, alkylamino, alkyl, carboxylic acid, ester, amide, carbonyl, alkoxyl, or cyano.

A large number of bile acids are known in the art and are amenable to the present invention. Bile acids occur in conjugation with glycine or taurine in bile of most vertebrates and some of them find use in medicine. Thus, some bile acids--due to their inherent pharmacological properties--are used as cholerectics (see, for example, James E. F. Reynolds (editor) Martindale The Extra Pharmacopoeia, 30th Edition, The Pharmaceutical Press, London (1993), page 1341). Representative examples of bile acids include cholic acid, deoxycholic acid, taurocholic acid, glycocholic acid, glycodeoxycholic acid, taurodeoxycholic acid, ursodeoxycholic acid, and chenodeoxycholic acid. Additional bile acids amenable to the present invention include those described in U.S. Patents 5,641,767; 5,656,277; 5,610,151; 5,428,182; and 3,910,888.

A large number of lipids are known in the art and are amenable to the present invention. Representative examples of lipids include lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, triacylglycerols, phosphoacylglycerols, sphingolipids, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, and tetraterpenes.

A large number of aromatic compounds are known in the art and are amenable to the present invention. Representative examples of aromatic compounds include optionally substituted phenyl, naphthyl, anthracenyl, phenanthrenyl, pyrenyl, pyridinyl,

quinolinyl, acridinyl, phenathridinyl, pyrazinyl, pyrimidinyl, pyridazinyl, quinoxalinyl, quinazolinyl, 1,7-phenanthrolinyl, indolyl, thianaphthenyl, benzoxazolyl, benzofuranyl, 1,2-benzisoxazolyl, benzimidazolyl, pyrrolyl, thiophenyl, isoxazolyl, pyrazolyl, thiazolyl, imidazolyl, tetrazolyl, and furanyl.

A large number of carbohydrates are known in the art and are amenable to the present invention. Representative examples of carbohydrates include erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, and talose; or a disaccharide or trisaccharide formed via a 1,4 glycoside linkage between any of them. In certain instances, the carbohydrate is a hexose or pentose.

A large number of polycyclic compounds are known in the art and are amenable to the present invention. Representative classes of polycyclic compounds include bicyclic compounds wherein, the first and second ring are independently a 3, 4, 5, or 6-member saturated or unsaturated carbon ring containing 0, 1, 2, or 3 hetereoatoms selected from the group consisting of O, N, or S. In certain instances, the first ring is an aromatic ring. In certain instances, the second ring is an aromatic ring. In certain instances, both rings are saturated. In certain instances, the first ring contains no heteroatoms. In certain instances, the second ring contains to heteroatoms. In certain instances, the first ring contains a nitrogen atom. In certain instances, the second ring contains a nitrogen atom. In certain instances, the polycyclic compound is a tricyclic compound, wherein the first, second, and third ring are independently a 3, 4, 5, or 6-member saturated or unsaturated carbon ring containing 0, 1, 2, or 3 hetereoatoms selected from the group consisting of O, N, or S. In certain instances, the first ring is an aromatic ring. In certain instances, the second ring is an aromatic ring. In certain instances, the third ring is an aromatic ring. In certain instances, all three rings are saturated. In certain instances, the first ring contains no heteroatoms. In certain instances, the second ring contains to heteroatoms. In certain instances, the third ring contains to heteroatoms. In certain instances, the first ring contains a nitrogen atom. In certain instances, the second ring contains a nitrogen atom. In certain instances, the third ring contains a nitrogen atom. In certain instances, the polycyclic compound is a bridged

polycyclic compound. In certain instances, the polycyclic compound is a bicyclo[2.2.1]heptane, bicyclo[2.2.2]octane, bicyclo[3.2.1]octane, bicyclo[3.2.2]nonane, or bicyclo[3.3.1]nonane.

A large number of crown ethers are known in the art and are amenable to the present invention. Crown ethers are macrocyclic, polyether, neutral compounds containing 4-20 oxygen atoms each separated from the next by two or more carbon atoms. Macrocyclic polyethers have been found to form stable complexes with salts of alkali metals and other metals and ammonium salts; "Macrocyclic polyethers and their complexes", C. J. Pederson et al, Angew. Chem. Intern. Ed., Vol. 11, page 16, (1972) and U.S. Pat. Nos. 3,562,295 and 3,687,978. Since the stereo models of macrocyclic polyethers give a crown-like appearance, they are commonly designated as N-crown-M polyethers, wherein N is the total number of atoms in the polyether ring and M is the number of oxygen atoms in the polyether ring. Crown polyethers ranging in size from cyclic tetramers of ethylene oxide ([12]-crown-4) and propylene oxide ([16]-crown-4) to 60-membered polyether rings (dibenzo [60]-crown-20) have been reported. Preferred crown ethers include 12-crown-4, 15-crown-5, and 18-crown-6.

A large number of oligonucleotide intercalators are known in the art and are amenable to the present invention. One class of intercalators are DNA intercalators which bind noncovalently to duplex DNA and are characterized by a flat molecule which inserts between base pairs of the double helix of DNA. Representative examples of intercalators include *p*-carboxy methidium, *p*-carboxy ethidium, acridine and ellipticine.

A large number of oligonucleotide cleaver molecules are known in the art and are amenable to the present invention. A cleaver molecule is a compound that can sever an oligonucleotide strand. Bleomycin, a glycopeptide antibiotic, is known to bind to and cleave DNA in a reaction that depends on the presence of ferrous ion and molecular oxygen, "Bleomycin: Chemical, Biochemical and Biological Aspects"; Hecht, S. M., Ed.; Springer Verlag: New York, 1979; Sausville, E. A.; Peisach, J.; Horwitz, S. B. "Biochemistry" 1978, 17, 2740. Burger, R. M.; Peisach, J; Horwitz, S. B. "Life Sciences" 1981, 28, 715; and Lown, J. W.; Sim, S. F. "Biochem. Biophys. Res. Comm. " 1977, 77, 1150. The antitumor agent streptonigrin is also capable of causing single strand breaks in

DNA using oxygen and cuprous ion, Cone, R; Hasan, S. K.; Lown, J. W.; Morgan, A. R. "Can. J. Biochem." 1976, 54, 219. Recently, the 1-10 phenanthroline-cuprous complex has been shown to cleave DNA in the presence of oxygen, Sigman, D. S.; Graham, D. R.; D'Aurora, V.; Stern, A. M. "J. Biol. Chem." 1979, 254, 12269; Graham, D. R.; Marshall, L. E.; Reich, K. A.; Sigman, D. S. "J. Amer. Chem. Soc." 1980, 102, 5419; Marshall, L. E.; Graham, D. R.; Reich, K. A.; Sigman, D. S. "Biochemistry" 1981, 20, 244; and Que, B. G.; Downey, K. M.; So., A. G. "Biochemistry" 1980, 19, 5987. In addition, methidium, ethidium, and cisplatin are known to cleave oligonucleotide sequences.

Ligands in general can include therapeutic modifiers, *e.g.*, for enhancing uptake; diagnostic compounds or reporter groups *e.g.*, for monitoring distribution; cross-linking agents; nuclease-resistance conferring moieties; and natural or unusual nucleobases. General examples include lipophiles, lipids, steroids (*e.g.*, cholesterol, uvaol, hecigenin, diosgenin), terpenes (*e.g.*, triterpenes, *e.g.*, sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), vitamins (*e.g.*, folic acid, vitamin A, vitamin E, biotin, pyridoxal), carbohydrates, proteins, protein binding agents, integrin targeting molecules,polycationics, peptides, polyamines, and peptide mimics.

The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

iRNA AGENT STRUCTURE

The monomers described herein can be used to make oligonucleotides which are useful as iRNA agents, *e.g.*, RNA molecules, (double-stranded; single-stranded) that mediate RNAi, *e.g.*, with respect to an endogenous gene of a subject or to a gene of a pathogen. In most cases the iRNA agent will incorporate momomers described herein together with naturally occuring nucleosides or nucleotides or with other modified

nucleosides or nucleotides. The modified monomers can be present at any position in the iRNA agent, *e.g.*, at the terminii or in the middle region of an iRNA agent or in a duplex region or in an unpaired region. In a preferred embodiment iRNA agent can have any architecture, *e.g.*, architecture described herein. *E.g.*, it can be incorporated into an iRNA agent having an overhang structure, a hairpin or other single strand structure or a two-strand structure, as described herein.

An "RNA agent" as used herein, is an unmodified RNA, modified RNA, or nucleoside surrogate, all of which are defined herein (see, *e.g.*, the section below entitled RNA Agents). While numerous modified RNAs and nucleoside surrogates are described, preferred examples include those which have greater resistance to nuclease degradation than do unmodified RNAs. Preferred examples include those which have a 2' sugar modification, a modification in a single strand overhang, preferably a 3' single strand overhang, or, particularly if single stranded, a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

An "iRNA agent," as used herein, is an RNA agent which can, or which can be cleaved into an RNA agent which can, stimulate or inhibit an immune response, or have no effect on an immune response. An iRNA agent may also down regulate the expression of a target gene, preferably an endogenous or pathogen target RNA. While not wishing to be bound by theory, an iRNA agent that down regulates expression of a target gene may act by one or more of a number of mechanisms, including post-transcriptional cleavage of a target mRNA (sometimes referred to in the art as RNAi), or pre-transcriptional or pre-translational mechanisms. An iRNA agent can include a single strand or can include more than one strands, *e.g.*, it can be a double stranded iRNA agent. If the iRNA agent is a single strand it is particularly preferred that it include a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

For ease of exposition the term nucleotide or ribonucleotide is sometimes used herein in reference to one or more monomeric subunits of an RNA agent. It will be understood herein that the usage of the term "ribonucleotide" or "nucleotide" can, in the

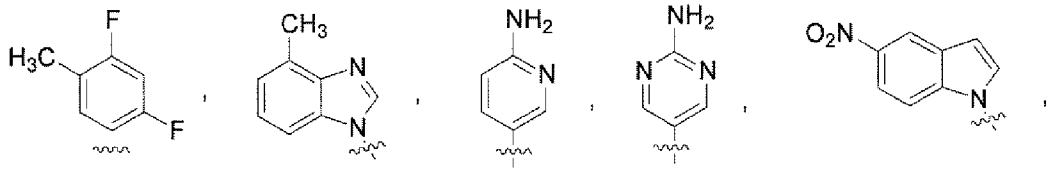
case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions.

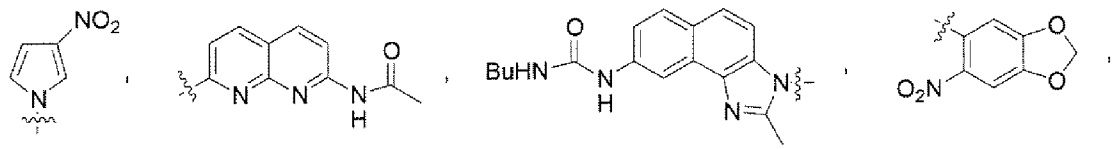
As discussed elsewhere herein, an iRNA agent will often be modified or include nucleoside surrogates in addition to the ribose replacement modification subunit (RRMS). Single stranded regions of an iRNA agent will often be modified or include nucleoside surrogates, *e.g.*, the unpaired region or regions of a hairpin structure, *e.g.*, a region which links two complementary regions, can have modifications or nucleoside surrogates. Modification to stabilize one or more 3'- or 5'-terminus of an iRNA agent, *e.g.*, against exonucleases, or to favor the antisense sRNA agent to enter into RISC are also favored. Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers, carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), special biotin or fluorescein reagents that come as phosphoramidites and that have another DMT-protected hydroxyl group, allowing multiple couplings during RNA synthesis.

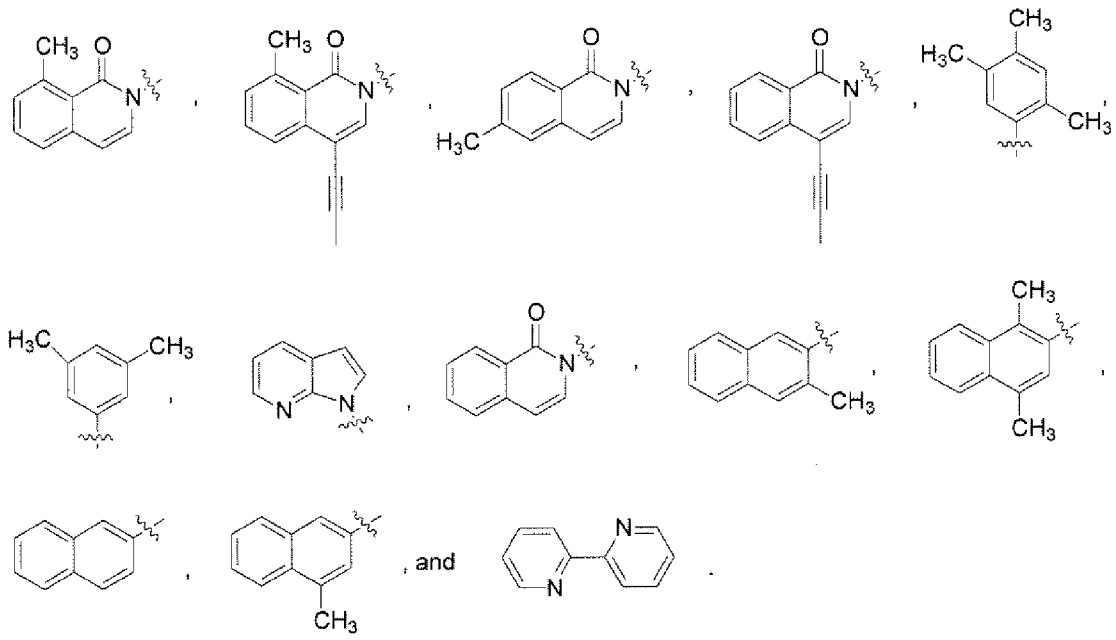
The Bases. Adenine, guanine, cytosine and uracil are the most common bases found in RNA. These bases can be modified or replaced to provide RNA's having improved properties. *E.g.*, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases, *e.g.*, "unusual bases" and "universal bases" described herein, can be employed. Examples include without limitation 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-

propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine,7-deazaadenine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases. Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

A universal base can form base pairs with each of the natural DNA/RNA bases, exhibiting relatively little discrimination between them. In general, the universal bases are non-hydrogen bonding, hydrophobic, aromatic moieties which can stabilize *e.g.*, duplex RNA or RNA-like molecules, via stacking interactions. A universal base can also include hydrogen bonding substituents. As used herein, a "universal base" can include anthracenes, pyrenes or any one of the following:







Generally, base changes are less preferred for promoting stability, but they can be useful for other reasons, *e.g.*, some, *e.g.*, 2,6-diaminopurine and 2 amino purine, are fluorescent. Modified bases can reduce target specificity. This should be taken into consideration in the design of iRNA agents.

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

48, 2223-2311 and Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1993**, *49*, 6123-6194, or references referred to therein.

Modification described in WO 00/44895, WO01/75164, or WO02/44321 can be used herein.

The disclosure of all publications, patents, and published patent applications listed herein are hereby incorporated by reference.

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

Sugar Group References. Modifications to the 2' modifications can be found in Manoharan, *Biochimica et Biophysica Acta* 1489:117-130, 1999; Verma, S. *et al. Annu. Rev. Biochem.* 67:99-134, 1998 and references therein. Specific modifications to the ribose can be found in the following references: 2'-fluoro (Kawasaki et. al., *J. Med. Chem.*, 1993, *36*, 831-841), 2'-MOE (Martin, P. *Helv. Chim. Acta* 1996, *79*, 1930-1938), "LNA" (Wengel, J. *Acc. Chem. Res.* 1999, *32*, 301-310). iRNA-specific chemical modifications are described in Manoharan, *Current Opinion in Chemical Biology* 8:570-579, 2004.

Cationic Groups

Modifications can also include attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate backbone

moiety. A cationic group can be attached to any atom capable of substitution on a natural, unusual or universal base. A preferred position is one that does not interfere with hybridization, *i.e.*, does not interfere with the hydrogen bonding interactions needed for base pairing. A cationic group can be attached *e.g.*, through the C2' position of a sugar or analogous position in a cyclic or acyclic sugar surrogate. Cationic groups can include *e.g.*, protonated amino groups, derived from *e.g.*, O-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); aminoalkoxy, *e.g.*, $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); amino (*e.g.* NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); or $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino).

Nonphosphate Linkages

Modifications can also include the incorporation of nonphosphate linkages at the 5' and/or 3' end of a strand. Examples of nonphosphate linkages which can replace the phosphate group include methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methyl phosphonate and hydroxylamino groups.

Modifications can also include linking two sugars via a phosphate or modified phosphate group through the 2' position of a first sugar and the 5' position of a second sugar. Also contemplated are inverted linkages in which both a first and second sugar are eached linked through the respective3' positions. Modified RNA's can also include "abasic" sugars, which lack a nucleobase at C-1'. The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified iRNA agent can include nucleotides

containing *e.g.*, arabinose, as the sugar. In another subset of this modification, the natural, unusual, or universal base may have the α-configuration. Modifcations can also include L-RNA.

Modifications can also include 5'-phosphonates, *e.g.*, $P(O)(O^-)_2$-X-$C^{5'}$-sugar (X= CH2, CF2, CHF and 5'-phosphate prodrugs, *e.g.*, $P(O)[OCH2CH2SC(O)R]_2CH_2C^{5'}$-sugar. In the latter case, the prodrug groups may be decomposed *via* reaction first with carboxy esterases. The remaining ethyl thiolate group via intramolecular S_N2 displacement can depart as episulfide to afford the underivatized phosphate group.

Modification can also include the addition of conjugating groups described elseqhere herein, which are prefereably attached to an iRNA agent through any amino group available for conjugation.

Nuclease resistant modifications include some which can be placed only at the terminus and others which can go at any position. Generally the modifications that can inhibit hybridization so it is preferably to use them only in terminal regions, and preferrable to not use them at the cleavage site or in the cleavage region of an sequence which targets a subject sequence or gene.. The can be used anywhere in a sense sequence, provided that sufficient hybridization between the two sequences of the iRNA agent is maintained. In some embodiments it is desirabable to put the NRM at the cleavage site or in the cleavage region of a sequence which does not target a subject sequence or gene,as it can minimize off-target silencing.

In addition, an iRNA agent described herein can have an overhang which does not form a duplex structure with the other sequence of the iRNA agent—it is an overhang, but it does hybridize, either with itself, or with another nucleic acid, other than the other sequence of the iRNA agent.

In most cases, the nuclease-resistance promoting modifications will be distributed differently depending on whether the sequence will target a sequence in the subject (often referred to as an anti-sense sequence) or will not target a sequence in the subject (often referred to as a sense sequence). If a sequence is to target a sequence in the subject, modifications which interfer with or inhibit endonuclease cleavage should not be inserted in the region which is subject to RISC mediated cleavage, *e.g.*, the cleavage site or the cleavage region (As described in Elbashir *et al.*, 2001, Genes and Dev. 15: 188, hereby

incorporated by reference, cleavage of the target occurs about in the middle of a 20 or 21 nt guide RNA, or about 10 or 11 nucleotides upstream of the first nucleotide which is complementary to the guide sequence. As used herein cleavage site refers to the nucleotide on either side of the cleavage site, on the target or on the iRNA agent strand which hybridizes to it. Cleavage region means an nucleotide with 1, 2, or 3 nucletides of the cleave site, in either direction.)

Such modifications can be introduced into the terminal regions, *e.g.*, at the terminal position or with 2, 3, 4, or 5 positions of the terminus, of a sequence which targets or a sequence which does not target a sequence in the subject.

An iRNA agent can have a first and a second strand chosen from the following:

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a first strand which does not target a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region;

a first strand which does not target a sequence and which has an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end; and

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a second strand which targets a sequence and which preferably does not have an an NRM modification at the cleavage site or in the cleavage region;

a second strand which targets a sequence and which does not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand).

An iRNA agent can also target two sequences and can have a first and second strand chosen from:

a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

a first strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a first strand which targets a sequence and which preferably does not have an an NRM modification at the cleavage site or in the cleavage region;

a first strand which targets a sequence and which dose not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5'

end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand) and

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end;

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end (5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand);

a second strand which targets a sequence and which has an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end and which has a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end;

a second strand which targets a sequence and which preferably does not have an an NRM modification at the cleavage site or in the cleavage region;

a second strand which targets a sequence and which dose not have an NRM modification at the cleavage site or in the cleavage region and one or more of an NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 3' end, a NRM modification at or within 1, 2, 3, 4, 5 , or 6 positions from the 5' end, or NRM modifications at or within 1, 2, 3, 4, 5 , or 6 positions from both the 3' and the 5' end(5' end NRM modifications are preferentially not at the terminus but rather at a position 1, 2, 3, 4, 5 , or 6 away from the 5' terminus of an antisense strand).

Ribose Mimics: The monomers and methods described herein can be used to prepare an RNA, *e.g.*, an iRNA agent, that incorporates a ribose mimic, such as those described herein and those described in copending co-owned United States Provisional Application Serial No. 60/454,962, filed on March 13, 2003, and International Application No. PCT/US04/07070, both of which are hereby incorporated by reference.

Thus, an aspect of the invention features an iRNA agent that includes a secondary hydroxyl group, which can increase efficacy and/or confer nuclease resistance to the agent. Nucleases, *e.g.*, cellular nucleases, can hydrolyze nucleic acid phosphodiester bonds, resulting in partial or complete degradation of the nucleic acid. The secondary hydroxy group confers nuclease resistance to an iRNA agent by rendering the iRNA agent less prone to nuclease degradation relative to an iRNA which lacks the

modification. While not wishing to be bound by theory, it is believed that the presence of a secondary hydroxyl group on the iRNA agent can act as a structural mimic of a 3' ribose hydroxyl group, thereby causing it to be less susceptible to degradation.

Combination therapy

In one aspect, compostion of the invention can be used in combination therapy. The term "combination therapy" includes the administration of the subject compounds in further combination with other biologically active ingredients (such as, but not limited to, a second and different antineoplastic agent) and non-drug therapies (such as, but not limited to, surgery or radiation treatment). For instance, the compounds of the invention can be used in combination with other pharmaceutically active compounds, preferably compounds that are able to enhance the effect of the compounds of the invention. The compounds of the invention can be administered simultaneously (as a single preparation or separate preparation) or sequentially to the other drug therapy. In general, a combination therapy envisions administration of two or more drugs during a single cycle or course of therapy.

In one aspect of the invention, the subject compounds may be administered in combination with one or more separate agents that modulate protein kinases involved in various disease states. Examples of such kinases may include, but are not limited to: serine/threonine specific kinases, receptor tyrosine specific kinases and non-receptor tyrosine specific kinases. Serine/threonine kinases include mitogen activated protein kinases (MAPK), meiosis specific kinase (MEK), RAF and aurora kinase. Examples of receptor kinase families include epidermal growth factor receptor (EGFR) (e.g. HER2/neu, HER3, HER4, ErbB, ErbB2, ErbB3, ErbB4, Xmrk, DER, Let23); fibroblast growth factor (FGF) receptor (e.g. FGF-Rl, GFF-R2/BEK/CEK3, FGF-R3/CEK2, FGF-R4/TKF, KGF-R); hepatocyte growth/scatter factor receptor (HGFR) (e.g, MET, RON, SEA, SEX); insulin receptor (e.g. IGFI-R); Eph (e.g. CEK5, CEK8, EBK, ECK, EEK, EHK-I, EHK-2, ELK, EPH, ERK, HEK, MDK2, MDK5, SEK); Axl (e.g. Mer/Nyk, Rse); RET; and platelet- derived growth factor receptor (PDGFR) (e.g. PDGFα-R, PDGβ-R, CSFl -R/FMS, SCF- R/C-KIT, VEGF-R/FLT, NEK/FLK1, FLT3/FLK2/STK-1). Non-receptor tyrosine kinase families include, but are not limited to, BCR-ABL (e.g.

p43^{abl}, ARG); BTK (e.g. ITK/EMT, TEC); CSK, FAK, FPS, JAK, SRC, BMX, FER, CDK and SYK.

In another aspect of the invention, the subject compounds may be administered in combination with one or more agents that modulate non-kinase biological targets or processes. Such targets include histone deacetylases (HDAC), DNA methyltransferase (DNMT), heat shock proteins (e.g. HSP90), and proteosomes.

In one embodiment, subject compounds may be combined with antineoplastic agents (e.g. small molecules, monoclonal antibodies, antisense RNA, and fusion proteins) that inhibit one or more biological targets such as Zolinza, Tarceva, Iressa, Tykerb, Gleevec, Sutent, Sprycel, Nexavar, Sorafinib, CNF2024, RG108, BMS387032, Affmitak, Avastin, Herceptin, Erbitux, AG24322, PD325901 , ZD6474, PD 184322,

Obatodax, ABT737 and AEE788. Such combinations may enhance therapeutic efficacy over efficacy achieved by any of the agents alone and may prevent or delay the appearance of resistant mutational variants.

In certain preferred embodiments, the compounds of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents encompass a wide range of therapeutic treatments in the field of oncology. These agents are administered at various stages of the disease for the purposes of shrinking tumors, destroying remaining cancer cells left over after surgery, inducing remission, maintaining remission and/or alleviating symptoms relating to the cancer or its treatment. Examples of such agents include, but are not limited to, alkylating agents such as mustard gas derivatives

(Mechlorethamine, cylophosphamide, chlorambucil, melphalan, ifosfamide), ethylenimines (thiotepa, hexamethylmelanine), Alkylsulfonates (Busulfan), Hydrazines and Triazines (Altretamine, Procarbazine, Dacarbazine and Temozolomide), Nitrosoureas (Carmustine, Lomustine and Streptozocin), Ifosfamide and metal salts (Carboplatin, Cisplatin, and Oxaliplatin); plant alkaloids such as Podophyllotoxins (Etoposide and Tenisopide), Taxanes (Paclitaxel and Docetaxel), Vinca alkaloids (Vincristine, Vinblastine, Vindesine and Vinorelbine), and Camptothecan analogs (Irinotecan and Topotecan); anti-tumor antibiotics such as Chromomycins (Dactinomycin and

Plicamycin), Anthracyclines (Doxorubicin, Daunorubicin, Epirubicin, Mitoxantrone, Valrubicin and Idarubicin), and miscellaneous antibiotics such as Mitomycin, Actinomycin and Bleomycin; anti-metabolites such as folic acid antagonists (Methotrexate, Pemetrexed, Raltitrexed, Aminopterin), pyrimidine antagonists (5-Fluorouracil, Floxuridine, Cytarabine, Capecitabine, and Gemcitabine), purine antagonists (6-Mercaptopurine and 6-Thioguanine) and adenosine deaminase inhibitors (Cladribine, Fludarabine, Mercaptopurine, Clofarabine, Thioguanine, Nelarabine and Pentostatin); topoisomerase inhibitors such as topoisomerase I inhibitors (Ironotecan, topotecan) and topoisomerase II inhibitors (Amsacrine, etoposide, etoposide phosphate, teniposide); monoclonal antibodies (Alemtuzumab, Gemtuzumab ozogamicin, Rituximab, Trastuzumab, Ibritumomab Tioxetan, Cetuximab, Panitumumab, Tositumomab, Bevacizumab); and miscellaneous anti-neoplasties such as ribonucleotide reductase inhibitors (Hydroxyurea); adrenocortical steroid inhibitor (Mitotane); enzymes (Asparaginase and Pegaspargase); anti-microtubule agents (Estramustine); and retinoids (Bexarotene, Isotretinoin, Tretinoin (ATRA). In certain preferred embodiments, the compounds of the invention are administered in combination with a chemoprotective agent. Chemoprotective agents act to protect the body or minimize the side effects of chemotherapy. Examples of such agents include, but are not limited to, amfostine, mesna, and dexrazoxane.

In one aspect of the invention, the subject compounds are administered in combination with radiation therapy. Radiation is commonly delivered internally (implantation of radioactive material near cancer site) or externally from a machine that employs photon (x-ray or gamma-ray) or particle radiation. Where the combination therapy further comprises radiation treatment, the radiation treatment may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of the therapeutic agents and radiation treatment is achieved. For example, in appropriate cases, the beneficial effect is still achieved when the radiation treatment is temporally removed from the administration of the therapeutic agents, perhaps by days or even weeks.

It will be appreciated that compounds of the invention can be used in combination with an immunotherapeutic agent. One form of immunotherapy is the generation of an active systemic tumor-specific immune response of host origin by administering a vaccine composition at a site distant from the tumor. Various types of vaccines have been proposed, including isolated tumor-antigen vaccines and anti-idiotype vaccines. Another approach is to use tumor cells from the subject to be treated, or a derivative of such cells (reviewed by Schirrmacher et al. (1995) J. Cancer Res. Clin. Oncol. 121 :487). In U.S. Pat. No. 5,484,596, Hanna Jr. et al. claim a method for treating a resectable carcinoma to prevent recurrence or metastases, comprising surgically removing the tumor, dispersing the cells with collagenase, irradiating the cells, and vaccinating the patient with at least three consecutive doses of about 10^7 cells.

It will be appreciated that the compounds of the invention may advantageously be used in conjunction with one or more adjunctive therapeutic agents. Examples of suitable agents for adjunctive therapy include steroids, such as corticosteroids (amcinonide, betamethasone, betamethasone dipropionate, betamethasone valerate, budesonide, clobetasol, clobetasol acetate, clobetasol butyrate, clobetasol 17-propionate, cortisone, deflazacort, desoximetasone, diflucortolone valerate, dexamethasone, dexamethasone sodium phosphate, desonide, furoate, fluocinonide, fluocinolone acetonide, halcinonide, hydrocortisone, hydrocortisone butyrate, hydrocortisone sodium succinate, hydrocortisone valerate, methyl prednisolone, mometasone, prednicarbate, prednisolone, triamcinolone, triamcinolone acetonide, and halobetasol proprionate); a 5HTi agonist, such as a triptan (e.g. sumatriptan or naratriptan); an adenosine Al agonist; an EP ligand; an NMDA modulator, such as a glycine antagonist; a sodium channel blocker (e.g. lamotrigine); a substance P antagonist (e.g. an NKi antagonist); a cannabinoid; acetaminophen or phenacetin; a 5 -lipoxygenase inhibitor; a leukotriene receptor antagonist; a DMARD (e.g. methotrexate); gabapentin and related compounds; a tricyclic antidepressant (e.g. amitryptilline); a neurone stabilising antiepileptic drug; a mono-aminergic uptake inhibitor (e.g. venlafaxine); a matrix metalloproteinase inhibitor; a nitric oxide synthase (NOS) inhibitor, such as an iNOS or an nNOS inhibitor; an inhibitor of the release, or action, of tumour necrosis factor α; an antibody therapy, such as a monoclonal antibody therapy; an antiviral agent, such as a nucleoside inhibitor (e.g.

lamivudine) or an immune system modulator (e.g. interferon); an opioid analgesic; a local anaesthetic; a stimulant, including caffeine; an H2-antagonist (e.g. ranitidine); a proton pump inhibitor (e.g. omeprazole); an antacid (e.g. aluminium or magnesium hydroxide; an antiflatulent (e.g. simethicone); a decongestant (e.g. phenylephrine, phenylpropanolamine, pseudoephedrine, oxymetazoline, epinephrine, naphazoline, xylometazoline, propylhexedrine, or levo-desoxyephedrine); an antitussive (e.g. codeine, hydrocodone, carmiphen, carbetapentane, or dextramethorphan); a diuretic; or a sedating or non-sedating antihistamine.

Pharmaceutical compositions. In one embodiment, the invention relates to a pharmaceutical composition containing an iRNA agent of the present invention, as described in the preceding sections, and a pharmaceutically acceptable carrier, as described below. A pharmaceutical composition including the modified iRNA agent is useful for treating a disease caused by expression of a target gene. In this aspect of the invention, the iRNA agent of the invention is formulated as described below. The pharmaceutical composition is administered in a dosage sufficient to inhibit expression of the target gene.

The pharmaceutical compositions of the present invention are administered in dosages sufficient to inhibit the expression or activity of the target gene. Compositions containing the iRNA agent of the invention can be administered at surprisingly low dosages. A maximum dosage of 5 mg iRNA agent per kilogram body weight per day may be sufficient to inhibit or completely suppress the expression or activity of the target gene.

In general, a suitable dose of modified iRNA agent will be in the range of 0.001 to 500 milligrams per kilogram body weight of the recipient per day (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 100 milligrams per kilogram, about 1 milligrams per kilogram to about 75 milligrams per kilogram, about 10 micrograms per kilogram to about 50 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram). The pharmaceutical composition may be administered once per day, or the iRNA agent may be administered as two, three, four, five, six or more sub-doses at appropriate intervals throughout the day. In that case, the iRNA agent contained in each

sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, *e.g.*, using a conventional sustained release formulation which provides sustained release of the iRNA agent over a several day period. Sustained release formulations are well known in the art. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the infection or disease, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and in vivo half-lives for the individual iRNA agent encompassed by the invention can be made using conventional methodologies or on the basis of in vivo testing using an appropriate animal model, as described elsewhere herein.

Advances in mouse genetics have generated a number of mouse models for the study of various human diseases. For example, mouse repositories can be found at The Jackson Laboratory, Charles River Laboratories, Taconic, Harlan, Mutant Mouse Regional Resource Centers (MMRRC) National Network and at the European Mouse Mutant Archive. Such models may be used for in vivo testing of iRNA agent, as well as for determining a therapeutically effective dose.

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

For oral administration, the iRNA agent useful in the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.

Tablets for oral use may include the active ingredients mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

Capsules for oral use include hard gelatin capsules in which the active ingredient is mixed with a solid diluent, and soft gelatin capsules wherein the active ingredient is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, the pharmaceutical compositions of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. In a preferred embodiment, the carrier consists exclusively of an aqueous buffer. In this context, "exclusively" means no auxiliary agents or encapsulating substances are present which might affect or mediate uptake of iRNA agent in the cells that harbor the target gene or virus. Such substances include, for example, micellar structures, such as liposomes or capsids, as described below. Although microinjection, lipofection, viruses, viroids, capsids, capsoids, or other auxiliary agents are required to introduce iRNA agent into cell cultures, surprisingly these methods and agents are not necessary for uptake of iRNA agent in vivo. The iRNA agent of the present invention are particularly advantageous in that they do not require the use of an auxiliary agent to mediate uptake of the iRNA agent into the cell, many of which agents are toxic or associated with deleterious side effects. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The pharmaceutical compositions can also include encapsulated formulations to protect the iRNA agent against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811; PCT publication WO 91/06309; and European patent publication EP-A-43075, which are incorporated by reference herein.

Toxicity and therapeutic efficacy of iRNA agent can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. iRNA agents that exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosages of compositions of the invention are preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any iRNA agent used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range of the iRNA agent or, when appropriate, of the polypeptide product of a target sequence (*e.g.*, achieving a decreased concentration of the polypeptide) that includes the IC50 (*i.e.*, the concentration of the test iRNA agent which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such

information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In addition to their administration individually or as a plurality, as discussed above, iRNA agents relating to the invention can be administered in combination with other known agents effective in treating viral infections and diseases. In any event, the administering physician can adjust the amount and timing of iRNA agent administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

For oral administration, the iRNA agent useful in the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.

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

Methods for identifying iRNA agents having increased stability. In yet another aspect, the invention relates to methods for identifying iRNA agent having increased stability in biological tissues and fluids such as serum. iRNA agent having increased stability have enhanced resistance to degradation, *e.g.*, by chemicals or nucleases (particularly endonucleases) which normally degrade RNA molecules. Methods for detecting increases in nucleic acid stability are well known in the art. Any assay capable of measuring or detecting differences between a test iRNA agent and a control iRNA agent in any measurable physical parameter may be suitable for use in the methods of the present invention. In general, because the inhibitory effect of an iRNA agent on a target gene activity or expression requires that the molecule remain intact, the stability of a particular iRNA agent can be evaluated indirectly by observing or measuring a property associated with the expression of the gene. Thus, the relative stability of an iRNA agent

can be determined by observing or detecting (1) an absence or observable decrease in the level of the protein encoded by the target gene, (2) an absence or observable decrease in the level of mRNA product from the target gene, and (3) a change or loss in phenotype associated with expression of the target gene. In the context of a medical treatment, the stability of an iRNA agent may be evaluated based on the degree of the inhibition of expression or function of the target gene, which in turn may be assessed based on a change in the disease condition of the patient, such as reduction in symptoms, remission, or a change in disease state.

In one embodiment, the method includes preparing an iRNA agent as described above (*e.g.*, through chemical synthesis), incubating the iRNA agent with a biological sample, then analyzing and identifying those iRNA agent that exhibit an increased stability as compared to a control iRNA agent.

In an exemplified embodiment, iRNA agent is produced in vitro by mixing/annealing complementary single-stranded RNA strands, preferably in a molar ratio of at least about 3:7, more preferably in a molar ratio of about 4:6, and most preferably in essentially equal molar amounts (*e.g.*, a molar ratio of about 5:5). Preferably, the single-stranded RNA strands are denatured prior to mixing/annealing, and the buffer in which the mixing/annealing reaction takes place contains a salt, preferably potassium chloride. Single-stranded RNA strands may be synthesized by solid phase synthesis using, for example, an Expedite 8909 synthesizer (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany), as described above.

iRNA agent are incubated with a biological sample under the conditions sufficient or optimal for enzymatic function. After incubating with a biological sample, the stability of the iRNA agent is analyzed by means conventional in the art, for example using RNA gel electrophoresis as exemplified herein. For example, when the sample is serum, the iRNA agent may be incubated at a concentration of 1-10 μM, preferably 2-8 μM, more preferably 3-6 μM, and most preferably 4-5 μM. The incubation temperature is preferably between 25°C and 45°C, more preferably between 35°C and 40°C, and most preferably about 37°C.

The biological sample used in the incubation step may be derived from tissues, cells, biological fluids or isolates thereof. For example, the biological sample may be

isolated from a subject, such as a whole organism or a subset of its tissues or cells. The biological sample may also be a component part of the subject, such as a body fluid, including but not limited to blood, serum, plasma, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen. Preferably, the biological sample is a serum derived from a blood sample of a subject. The subject is preferably a mammal, more preferably a human or a mouse.

In another embodiment, the method includes selecting an iRNA agent having increased stability by measuring the mRNA and/or protein expression levels of a target gene in a cell following introduction of the iRNA agent. In this embodiment, an iRNA agent of the invention inhibits expression of a target gene in a cell, and thus the method includes selecting an iRNA agent that induces a measurable reduction in expression of a target gene as compared to a control iRNA agent. Assays that measure gene expression by monitoring RNA and/or protein levels can be performed within about 24 hours following uptake of the iRNA agent by the cell. For example, RNA levels can be measured by Northern blot techniques, RNAse Protection Assays, or Quality Control-PCR (QC-PCR) (including quantitative reverse transcription coupled PCR (RT-PCR)) and analogous methods known in the art. Protein levels can be assayed, for example, by Western blot techniques, flow cytometry, or reporter gene expression (*e.g.*, expression of a fluorescent reporter protein, such as green fluorescent protein (GFP)). RNA and/or protein levels resulting from target gene expression can be measured at regular time intervals following introduction of the test iRNA agent, and the levels are compared to those following introduction of a control iRNA agent into cells. A control iRNA agent can be a nonsensical iRNA agent (*i.e.*, an iRNA agent having a scrambled sequence that does not target any nucleotide sequence in the subject), an iRNA agent that can target a gene not present in the subject (*e.g.*, a luciferase gene, when the iRNA agent is tested in human cells), or an iRNA agent otherwise previously shown to be ineffective at silencing the target gene. The mRNA and protein levels of the test sample and the control sample can be compared. The test iRNA agent is selected as having increased stability when there is a measurable reduction in expression levels following absorption of the test iRNA agent as compared to the control iRNA agent. mRNA and protein measurements can be made using any art-recognized technique (see, *e.g.*, Chiang, M.Y., *et al.*, *J. Biol*

Chem. (1991) 266:18162-71; Fisher, T, *et al.*, *Nucl. Acids Res.* (1993) 21:3857; and Chen *et al., J. Biol. Chem.* (1996) 271:28259).

Methods for identifying iRNA agents with ability to inhibit gene expression. The ability of an iRNA agent composition of the invention to inhibit gene expression can be measured using a variety of techniques known in the art. For example, Northern blot analysis can be used to measure the presence of RNA encoding a target protein. The level of the specific mRNA produced by the target gene can be measured, *e.g.*, using RT-PCR. Because iRNA agent directs the sequence-specific degradation of endogenous mRNA through RNAi, the selection methods of the invention encompass any technique that is capable of detecting a measurable reduction in the target RNA. In yet another example, Western blots can be used to measure the amount of target protein present. In still another embodiment, a phenotype influenced by the amount of the protein can be detected. Techniques for performing Western blots are well known in the art (see, *e.g.*, Chen, *et al.*, *J. Biol. Chem.* (1996) 271:28259).

When the target gene is to be silenced by an iRNA agent that targets a promoter sequence of the target gene, the target gene can be fused to a reporter gene, and reporter gene expression (*e.g.*, transcription and/or translation) can be monitored. Similarly, when the target gene is to be silenced by an iRNA agent that targets a sequence other than a promoter, a portion of the target gene (*e.g.*, a portion including the target sequence) can be fused with a reporter gene so that the reporter gene is transcribed. By monitoring a change in the expression of the reporter gene in the presence of the iRNA agent, it is possible to determine the effectiveness of the iRNA agent in inhibiting the expression of the reporter gene. The expression levels of the reporter gene in the presence of the test iRNA agent versus a control iRNA agent are then compared. The test iRNA agent is selected as having increased stability when there is a measurable reduction in expression levels of the reporter gene as compared to the control iRNA agent. Examples of reporter genes useful for use in the present invention include, without limitation, those coding for luciferase, GFP, chloramphenicol acetyl transferase (CAT), β-galactosidase, and alkaline phosphatase. Suitable reporter genes are described, for example, in *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (Ausubel, F.A., *et al.*, eds., 1989); Gould, S. J., and S. Subramani, *Anal. Biochem.* (1988) 7:404-408; Gorman, C. M., *et al.*,

Mol. Cell. Biol. (1982) 2:1044-1051; and Selden, R., *et al.*, *Mol. Cell. Biol.* (1986) 6:3173-3179; each of which is hereby incorporated by reference.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

SYNTHETIC METHODS

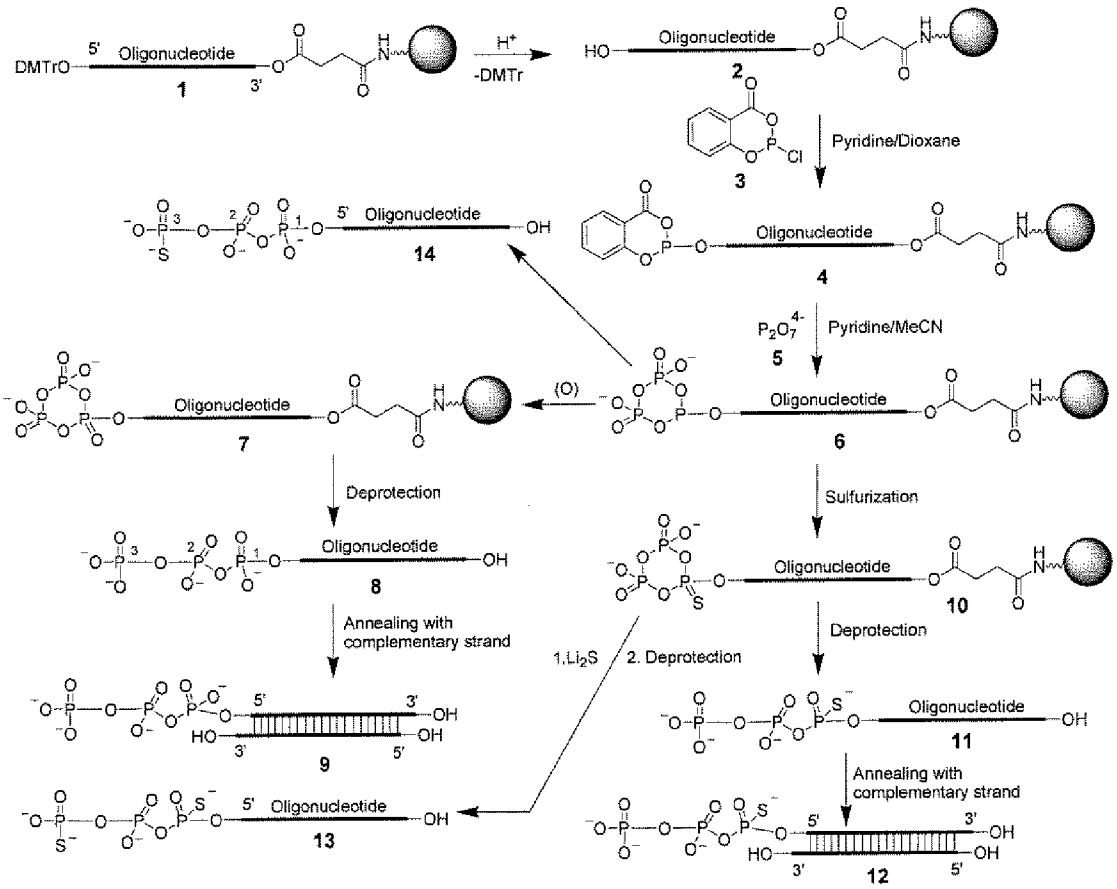
The iRNA agents of the present invention can be prepared according to standard procedures known in the art such as in the following references which are incorporated by the reference by its entirety:

1. *Chemical synthesis of oligonucleotide triphosphates.* Vaghefi, M. M.. Editor(s): Vaghefi, Morteza. *Nucleoside Triphosphates and Their Analogs*, **2005**, 105-113.

2. *Solid phase synthesis of 5'-diphosphorylated oligoribonucleotides and their conversion to capped m7Gppp-oligoribonucleotides for use as primers for influenza A virus RNA polymerase in vitro.* Brownlee, G. G.; Fodor, E.; Pritlove, D. C.; Gould, K. G.; Dalluge, J. J., *Nucleic Acids Res.* **1995**, *23(14)*, 2641-7.
3. *Chemical synthesis of 5'-pyrophosphate and triphosphate derivatives of 3',5' ApA, ApG, GpA and GpG. CD study of the effect of 5'-phosphate groups on the conformation of 3',5' GpG.* Tomasz, J.; Simoncsits, A.; Kajtar, M.; Krug, R. M.; Shatkin, A. J., *Nucleic Acids Res.*, **1978**, *5(8)*, 2945-57.
4. *Chemical synthesis of an effective inhibitor or protein synthesis in euharytic cells: pppA2',5'A2',5'A and some analogues.* Hartog, J. A. J. den; Wijnands, R. A.; van Boom, J. H.; *Nucleic Acids Symp. Series No 7*, **1980**, 157-166.
5. *Rapid and efficient synthesis of nucleoside 5'-0-(1-thiotriphosphates), 5'-triphosphates and 2',3'-cyclophosphorothioates using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one.* Ludwig, Janos; Eckstein, Fritz., *J. Org. Chem.*, **1989**, *54(3)*, 631-35.
6. *Novel solid phase synthesis of 2'-O-methylribonucleoside 5'-triphosphates and their α-thio analogs.* Gaur, R. K.; Sproat, B. S.; Krupp, Guido, *Tetrahedron Lett.* **1992**, *33(23)*, 3301-4.

7. *Preparation of oligodeoxynucleotide 5'-triphosphates using solid support approach.* Lebedev, A. V.; Koukhareva, I. I.; Beck, T.; Vaghefi, M. M. *Nucleosides, Nucleotides & Nucleic Acids*, **2001**, *20(4-7)*,1403-1409.
8. *Synthesis and properties of NTP analogs with modified triphosphate chains.* Koukhareva, Inna; Vaghefi, Morteza; Lebedev, Alexandre. Editor(s): Vaghefi, M.*Nucleoside Triphosphates and Their Analogs*, **2005**, 39-104 (and references cited there in)
9. *Nucleoside Triphosphates and their Analogs: Chemistry, Biotechnology, and Biological Applications.* Vaghefi, M.; Editor. USA. **2005**, 393 pp. Publisher: CRC Press LLC, Boca Raton, Fla. (and references cited there in).

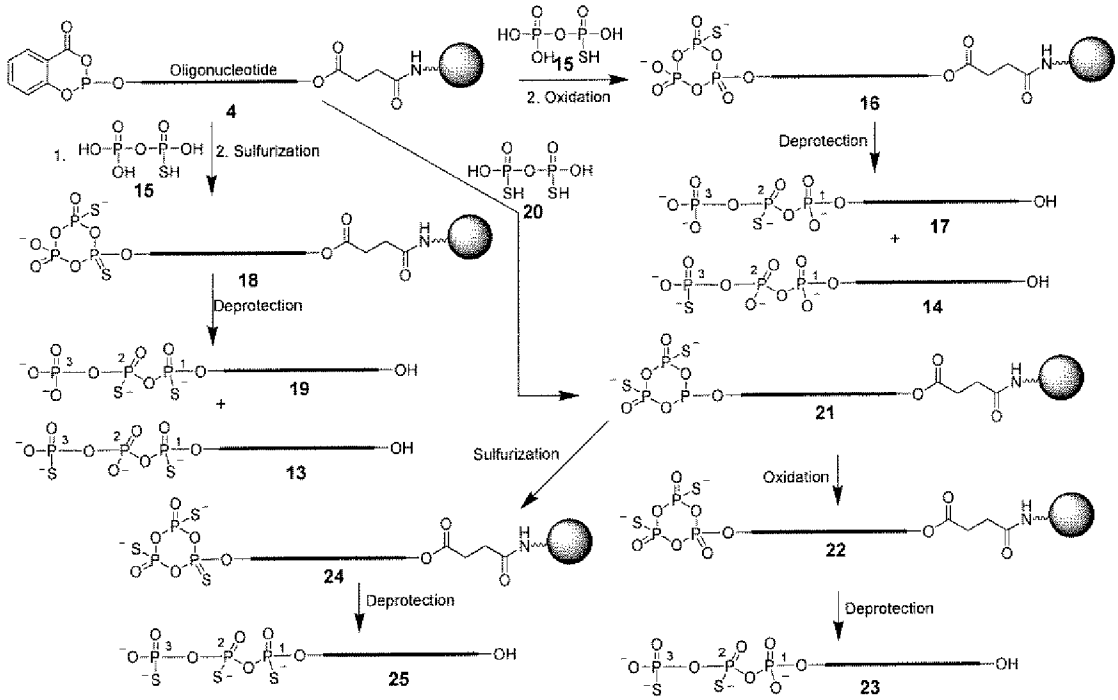
Scheme 1[a]



[a] Solid phase synthesis of oligonucleotide 5'-triphosphate, 5'-P1(thio) or P3(thio)-triphosphate and P(1), P(3)-dithiotriphospahte, and annealing with complementary strand. Detritylation: under standard solid oligonucleotide synthesis condition; Oxidation: I_2/H_2O

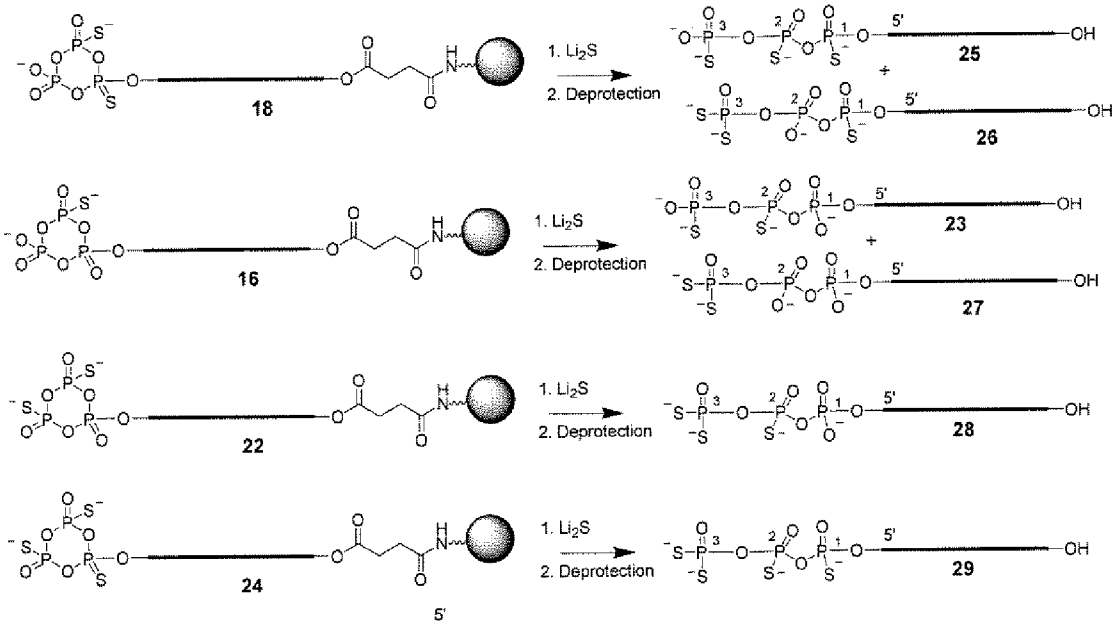
or milder oxidation agents such as *t*-butylhydroperoxide depends on the chemical modifications on the oligonucleotide; Sulfurization: Beaucage reagent (Thermo Scientific), DDTT (AM Chemicals) or other. Deprotection: for RNA oligonucleotide – 1(a) methylamine and 1(b) Py-HF or 2(a) ammonia and 2(b) Py-HF; for DNA oligonucleotide – 1. methylamine or 2. ammonia

Scheme 2[a]



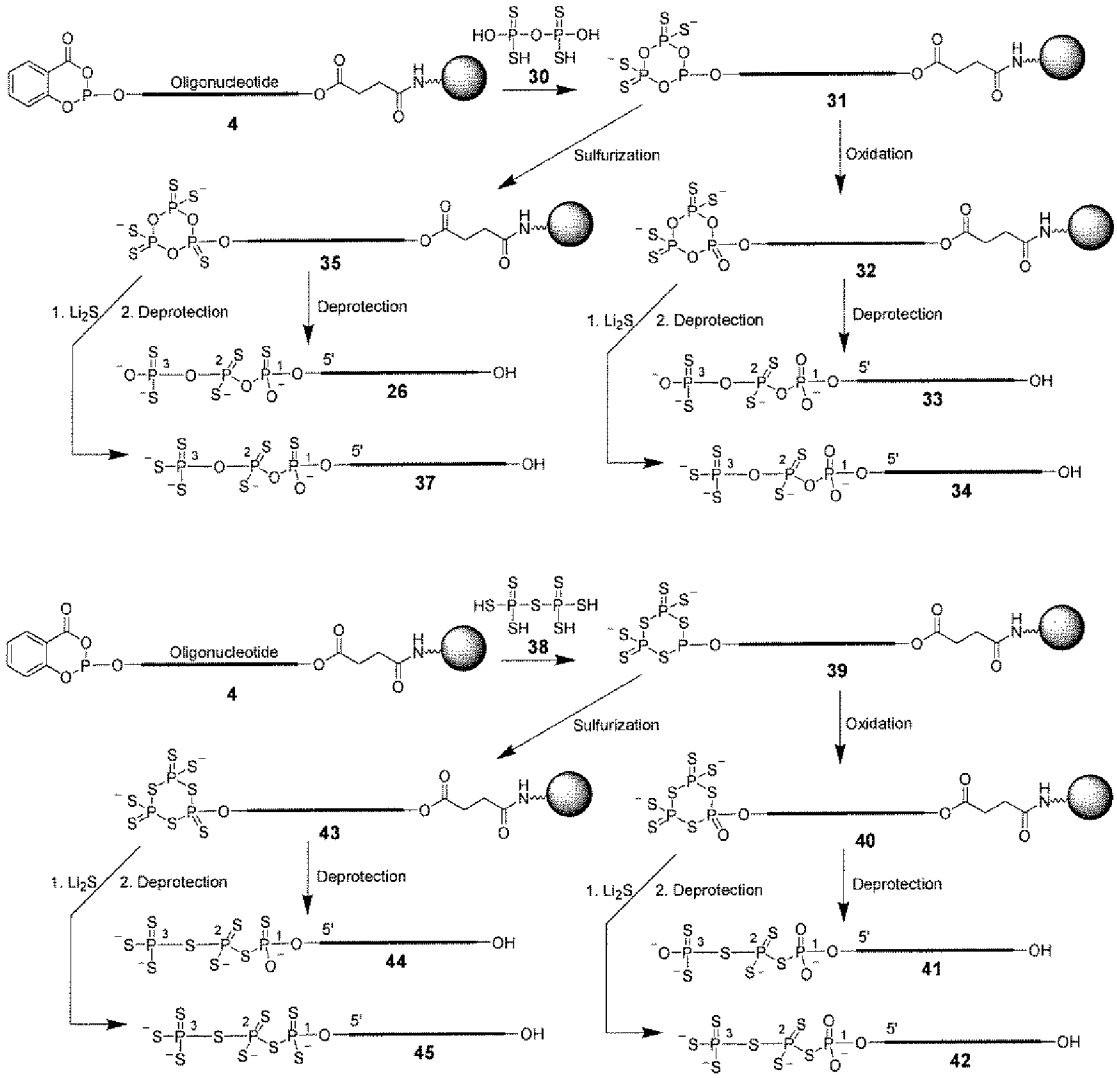
[a] Solid phase synthesis of oligonucleotide5'-P(1) or P(2) or P(3)-monothiotriphosaphate; 5'-P(1),P(2)- or P(1),P(3)- or P(2),P(3)-dithiotriphosphate and P(1),P(2),P(3)-trithiophosphate.

Scheme 3[a]



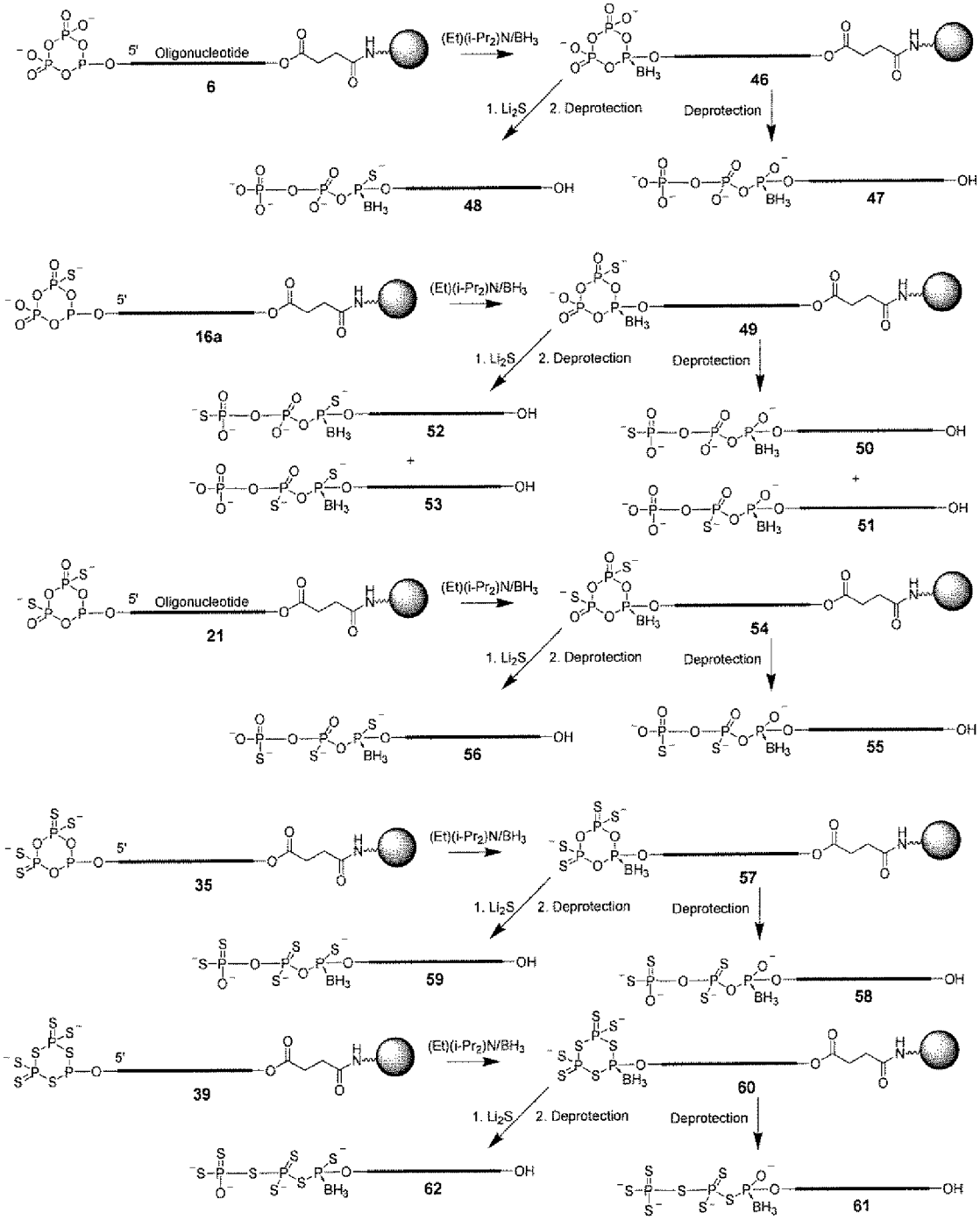
[a] Solid phase synthesis of Oligonucleotide 5'-P(3)-dithiophosphates

Scheme 4[a]



[a] Solid phase synthesis of oligonucleotide 5'-pyrothiophosphates

Scheme 5[a]



[a] Solid phase synthesis of oligonucleotide 5'-P(1)-boranotriphosphates and 5'-P(1)-borano-thiotriphosphates

EXAMPLES

RNA 5' triphosphate synthesis:

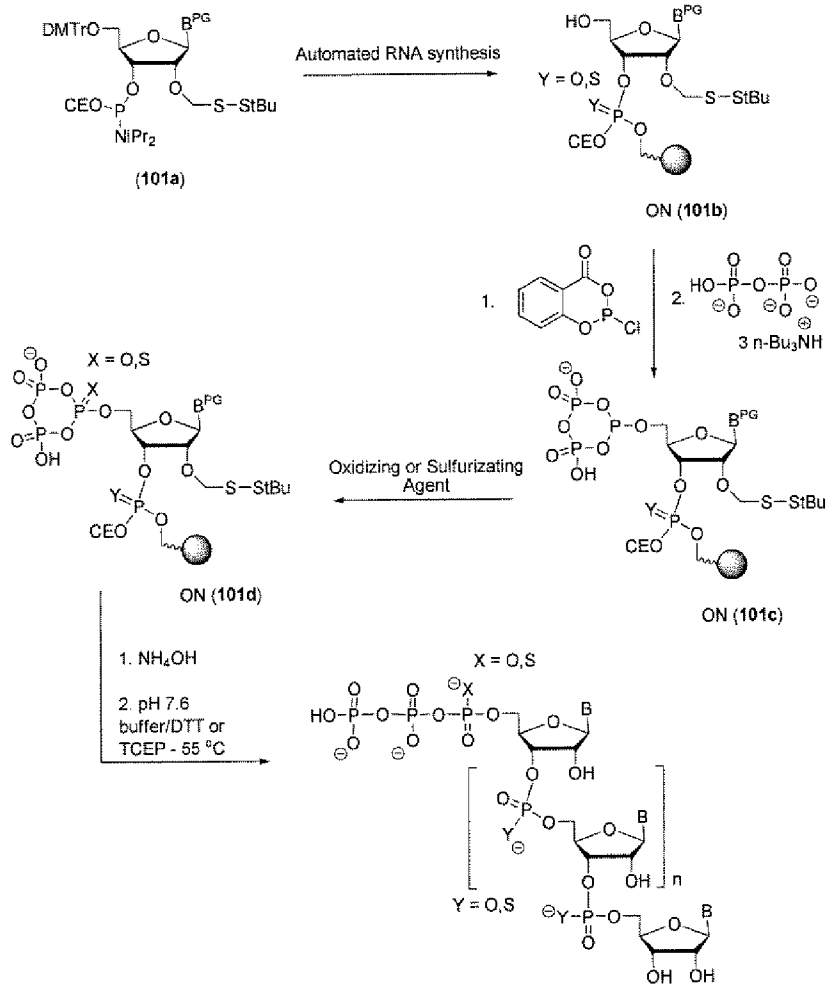
Syntheses of RNA 5' triphosphate involving various 2' O protecting group strategies as well as various chemistries for the introduction of the 5' triphosphate moiety or modified analog.

RNA 5'-triphosphates (RNATP) can be efficiently prepared in various manners involving alternate protecting groups for the 2' hydroxyls. All the 2' protecting groups developed and reported so far, (Reviewed by Beaucage, Cur. Op. Drug Disc. Dev., 2008, 11, 203) can be used for the synthesis of RNATP. These include and are not limited to the well known fluoro-labile groups such as TBDMS, TOM, CEM and others, but protecting groups that are cleaved in non fluoride ion conditions can also be of particular interest as well. This is because extended fluoride ion treatment and/or heating procedures can damage the RNATP and diminish yields. Likewise, extended purification/desalting procedures will have the same effect.

All these alternate syntheses involve the use of readily available 3' phosphoramidite building blocks (compounds a in the schemes) as monomer building blocks for automated RNA synthesis. The choice of protecting groups for the exocyclic amino groups of the nucleobases of these monomer building blocks (noted B^{PG} in the schemes) can also be modulated as base labile (fast labile) protecting groups are preferentially used (i.e. acetyl for C, phenoxyacetyl (Pac) for A and G and/or tert-butylphenoxyacetyl (tBuPac) and iso-propylphenoxyacetyl (iPrPac) for G. These fast labile protecting groups have the advantage to be removed without heating the reaction mixture, as it is needed for standard protecting groups. This later point improves the yields of target RNATP.

Examples of RNATP synthesis, presenting various 2' protecting groups, all using the Ludwig phosphitylation reagent (Ludwig and Ekcstein, 1989, *J. Org. Chem.*, 54, 631), are described.

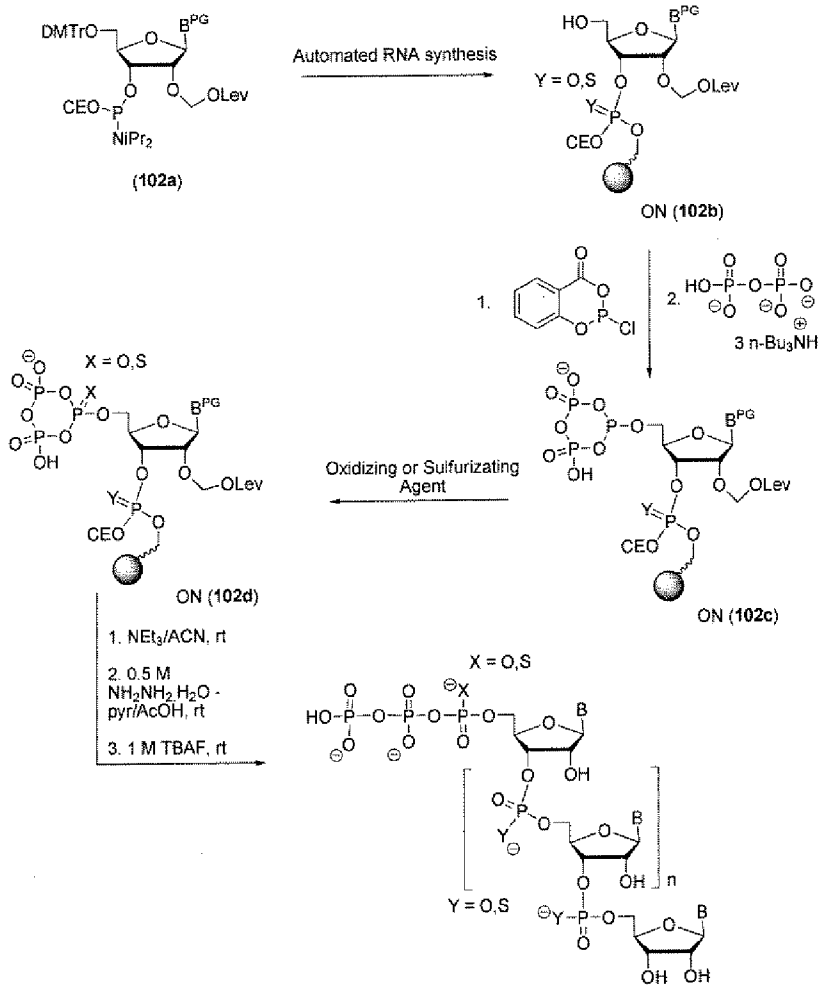
Scheme 6



Scheme 6: Target RNATP can be synthesized using the 2' tert-butyldithiomethyl (**DTM**) protecting group (Kwiatkowski et al., 2006, *J. Am. Chem. Soc.*, 128, 12356). The corresponding nucleobase protected (standard or fast labile PG) 5' DMTr 3' phosphoramidites (101a) are used in the automated RNA synthesis using the standard conditions on solid support (succinyl linked LCAA CPG). Other supports and linkers can be readily used as well. The resulting solid supported oligonucleotide, lacking the 5' terminal DMTr group (5' OH, compound 101b) is involved in a phosphitylation reaction using excess salicylophosphorochloridite in pyridine (Ludwig and Ekcstein, 1989, *J. Org. Chem.*, 54, 631). After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and

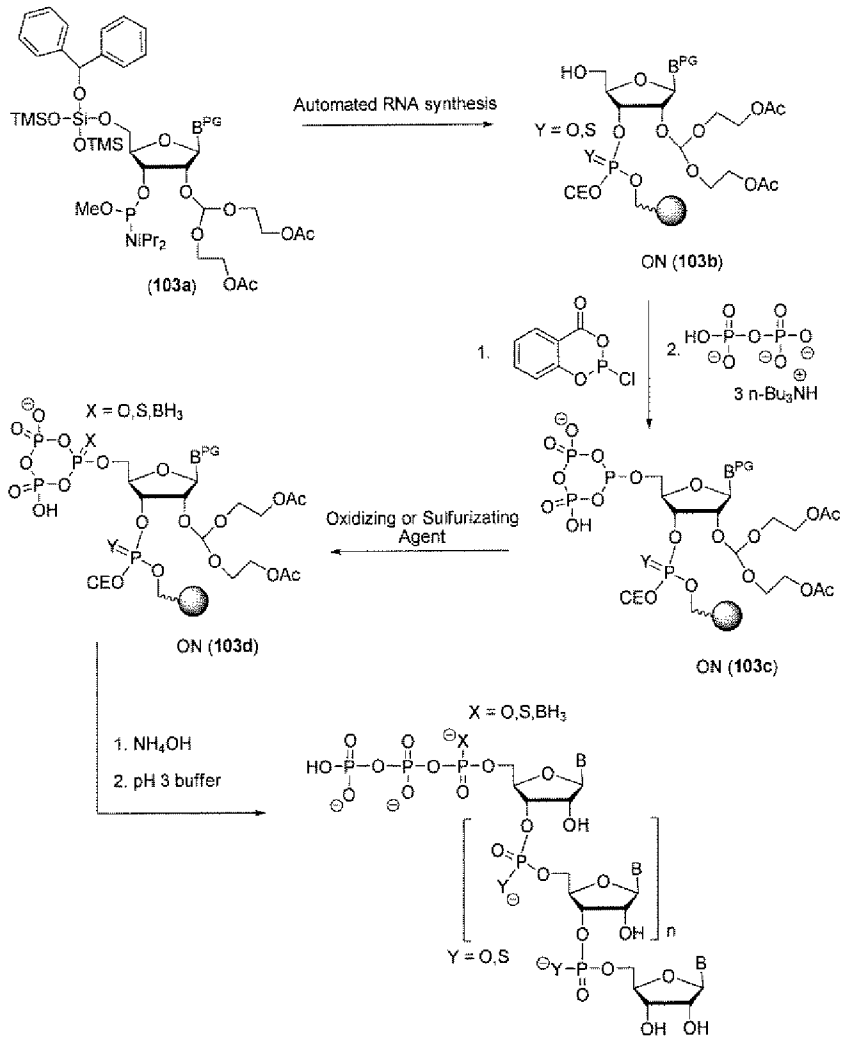
the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (101c) is oxidized or sulfurized using standard reagents. The corresponding cyclic TP (101d) is then hydrolyzed upon treatment with ammonia, allowing at the same time deblocking of the oligonucleotide from the solid support and nucleobase deprotection. Finally, removal of all the 2' DTM groups using a reducing agent such as DTT or TCEP in a pH 7.6 buffer at 55 °C provides the target RNATP or RNA(αS)TP.

Scheme 7



Scheme 7: Target RNATP can be synthesized using the 2' acetal levulinyl (**ALE**) protecting group (Damha et al, 2009, *J. Am. Chem. Soc*, 131, 8496). The corresponding base protected (PG = levulinyl for A and C, DMF for G), 5' DMTr 3' phosphoramidites (102a) are used in the automated RNA synthesis using the standard conditions on solid support (Q linker LCAA CPG is used with this 2' protecting group). Other supports and linkers can be possibly used as well. The resulting solid supported oligonucleotide, lacking the 5' terminal DMTr group (5' OH, compound 102b) is involved in a phosphitylation reaction using excess salicylophosphorochloridite in pyridine. After shaking the solid support with the phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (102c) is oxidized or sulfurized using standard reagents. The corresponding cyclic TP (102d) is then hydrolyzed by adding water in ACN, then rinsed with dry ACN and well dried under Argon flush. Particular deblocking conditions are used for the ALE group, i.e. firstly the backbone cyanoethyl groups are removed upon treatment with triethylamine in anhydrous acetonitrile. Then the 2' ALE groups, together with the base protecting groups are cleaved upon treatment with hydrazine hydrate in a mixture buffer of AcOH and pyridine. Finally cleavage of the Q linker with 1 M TBAF at room temperature provides the target RNATP or RNA(αS)TP.

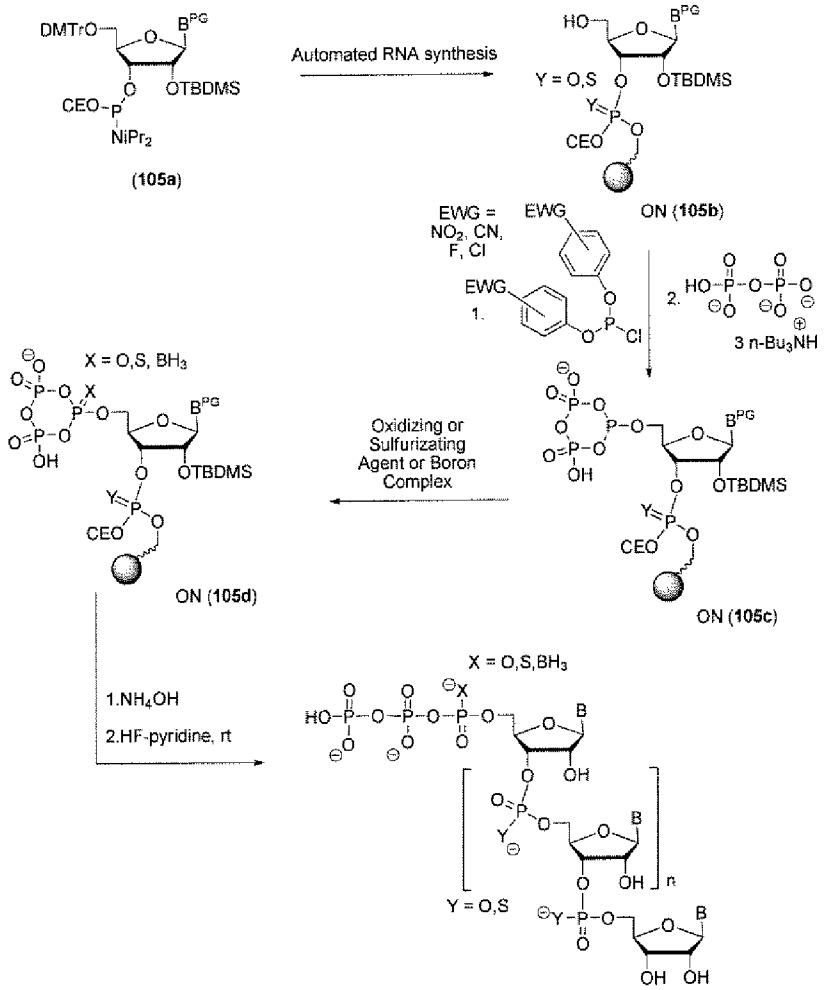
Scheme 8



Scheme 8: Target RNATP can be synthesized using the 2' bis acetoxyethoxymethyl (**ACE**) protecting group (Caruthers et al., 1998, *J. Am. Chem. Soc.*, 120, 11820). The corresponding base protected (standard or fast labile PG) 5' silyl (DOD) 3' phosphoramidites (103a) are commercially available and are used in the automated RNA synthesis using the standard conditions on solid support (succinyl linked LCAA CPG). Other supports and linkers can be readily used as well. The resulting solid supported oligonucleotide, lacking the 5' terminal silyl group (5' OH, compound 103b) is involved in a phosphitylation reaction using excess salicylophosphorochloridite in pyridine. After

shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (103c) is oxidized or sulfurized using standard reagents. The corresponding cyclic TP (103d) is then hydrolyzed upon treatment with ammonia, allowing at the same time deblocking of the oligonucleotide from the solid support and nucleobase deprotection. Finally, removal of all the 2' ACE groups using a slightly acidic pH 3 buffer provides the target RNATP or RNA(αS)TP.

Scheme 9



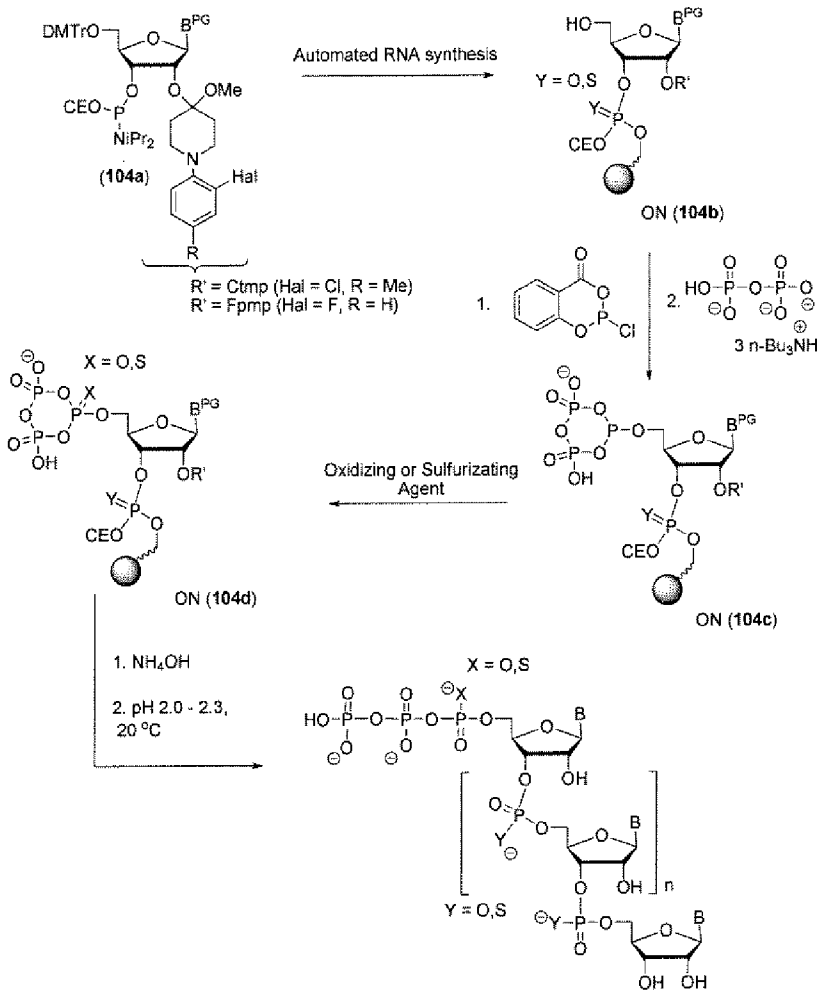
Scheme 9: Target RNATP can be synthesized using the acid labile 2' Ctmp or Fpmp protecting group (Reese et al., 1994, *Nucleic Acids Res.*, 22, 2209). The corresponding base protected (standard or fast labile PG) 5' DMTr 3' phosphoramidites (104a) are commercially available and used in the automated RNA synthesis using the standard conditions on solid support (succinyl linked LCAA CPG). Other supports and linkers can be readily used as well. The resulting solid supported oligonucleotide, lacking the 5' terminal DMTr group (5' OH, compound 104b) is involved in a phosphitylation reaction using excess salicylophosphorochloridite in pyridine. After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (104c) is oxidized or sulfurized using standard reagents. The corresponding cyclic TP (104d) is then hydrolyzed upon treatment with ammonia, allowing at the same time deblocking of the oligonucleotide from the solid support and nucleobase deprotection. Finally, removal of all the 2' Ctmp/Ftmp groups using an acidic pH 2.0 -2.3 buffer provides the target RNATP or RNA(αS)TP.

In combination with the variation of 2' protecting groups and/or nucleobase standard or fast labile protecting groups, several different ways, other than the Ludwig-Eckstein method, previously depicted, of introducing the triphosphate (or modified α-thio or α-borano analog) moiety to the solid supported oligonucleotide, can be applied. These include the use and the preparation of various 5' bis (mono or polysubstituted) aryl phosphite triesters, 5' phosphorodiamidites, 5'oxo thia or dithiaphospho-2-lanes or solid supported 5' mono(thio)phosphates. Some examples are described hereafter. For the synthesis of RNA 5' alpha thio triphosphates and 5' alpha dithio triphosphates, the use and preparation of 5' H-phosphonothioates and 5' H-phosphonodithioates, using diphenyl thiophosphite is described.

Schemes 5-8: In a general way, the salicyl part of the Ludwig reagent can be replaced by a bis aryl moiety, which is mono or polysubstituted by different electron withdrawal groups (EWG). Such EWG can be nitro, cyano, ortho-chloro, ortho-fluoro,

ortho,para di-chloro, ortho,para-di-flluoro, pentachloro, pentafluoro and others. The use of such O,O- bis aryl phosphorochloridites affords, after reaction with tributylammonium pyrophosphate, the cyclic intermediate (c). After the oxidizing/sulfurization or borane complexation step, followed by deprotection and support cleavage steps, the target RNATP or modified analog is obtained in similar fashion. Some examples exhibiting the various 2' protecting groups are depicted in schemes 5-8 and will be briefly described hereafter.

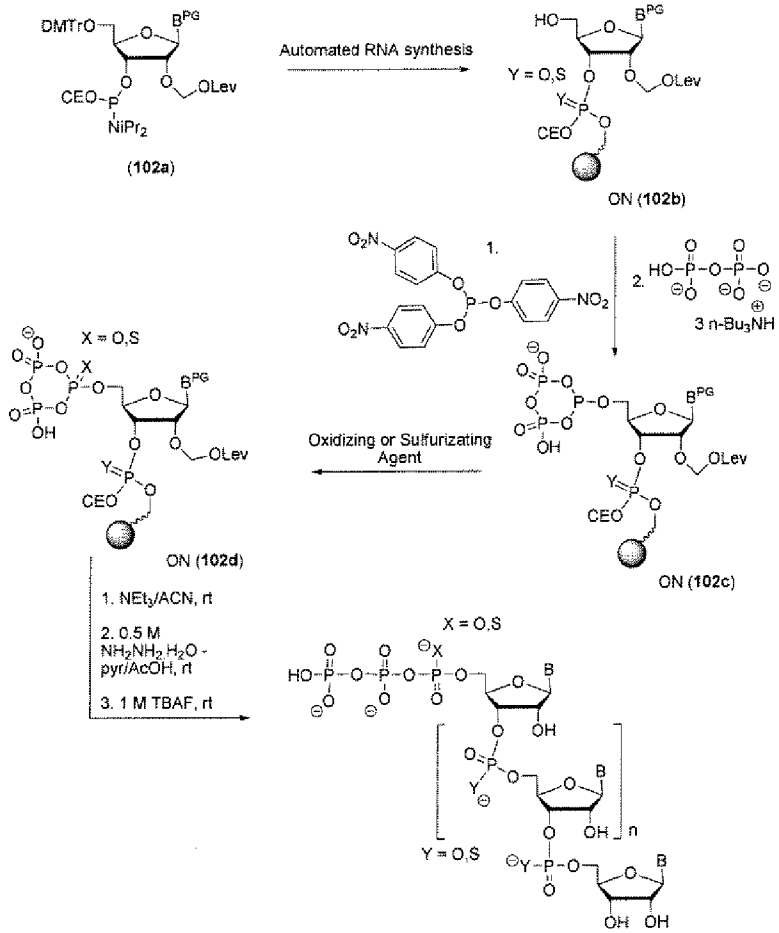
Scheme 10



Scheme 10: The most commonly used 2' TBDMS protected 5' DMTr 3' phosphoramidites (105a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 105b). It is then

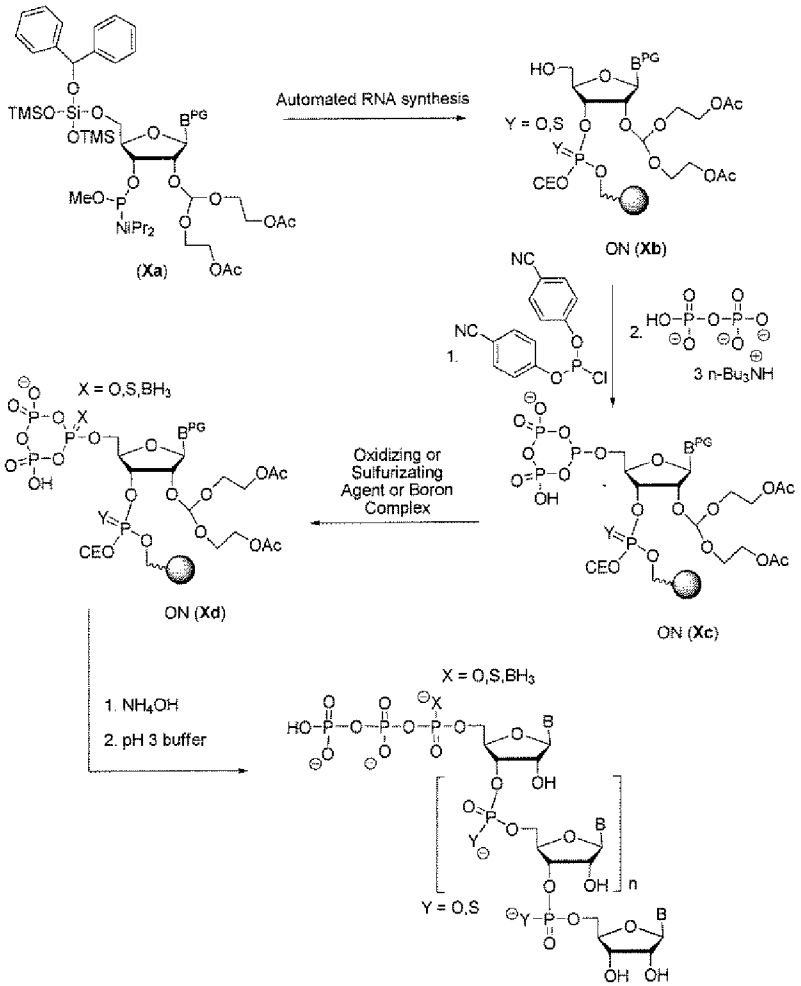
involved in a phosphitylation reaction using excess O,O- bis aryl phosphorochloridites in solution in dry pyridine. After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile and dried with Argon flux. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (105c) is oxidized, sulfurized or reacted with a suitable boron complex, using standard reagents. The corresponding cyclic TP (105d) is then hydrolyzed upon treatment with ammonia, allowing at the same time deblocking of the oligonucleotide from the solid support and nucleobase deprotection. Finally, removal of all the 2' TBDMS groups using HF-pyridine provides the target RNATP, RNA(αS)TP or RNA(αBH_3)TP.

Scheme 11



Scheme 11: The 2' ALE protected 5' DMTr 3' phosphoramidites (102a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 102b), as previously discussed for scheme 2. It is then involved in a phosphitylation reaction using excess O,O,O- tris 4-nitrophenyl phosphite in solution in dry pyridine. After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (102c) is oxidized, sulfurized or reacted with a suitable boron complex, using standard reagents. Previously described hydrolysis and deblocking steps of (102d) afford the target RNATP, RNA(αS)TP or RNA(αBH_3)TP.

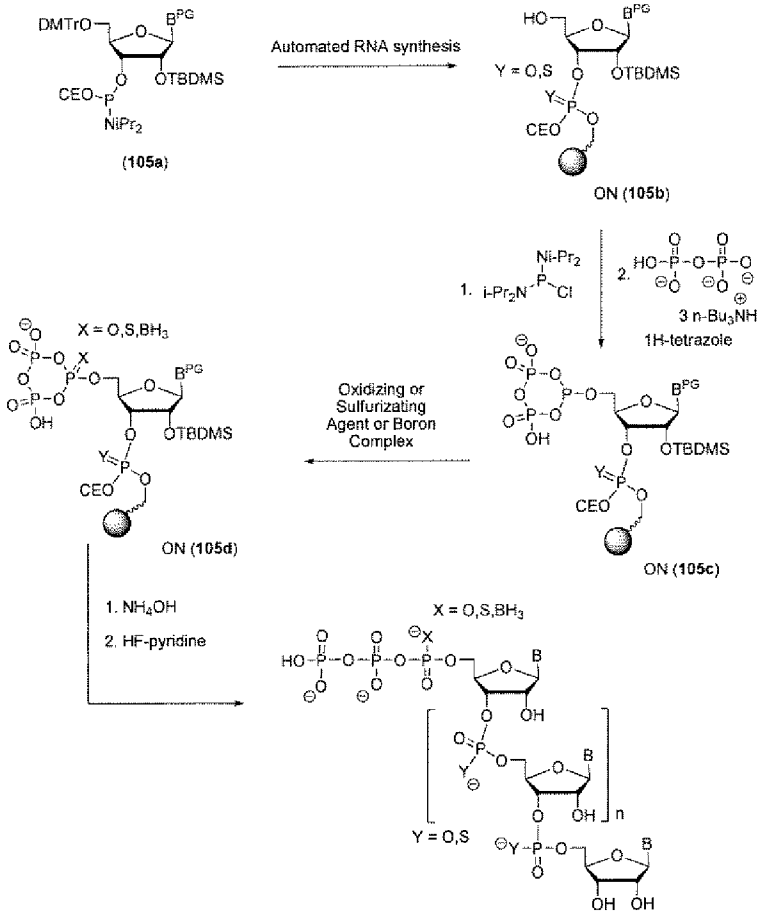
Scheme 12



Scheme 12: The 2' ACE protected phosphoramidites (103a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 103b), as previously discussed for scheme 3. It is then involved in a phosphitylation reaction using excess O,O- bis 4-cyano phosphorochloridite in solution in dry pyridine. After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (103c) is oxidized, sulfurized or reacted with a suitable boron complex, using standard reagents. Previously described

hydrolysis and deblocking steps of (103d) afford the target RNATP, RNA(αS)TP or RNA(αBH$_3$)TP.

Scheme 13

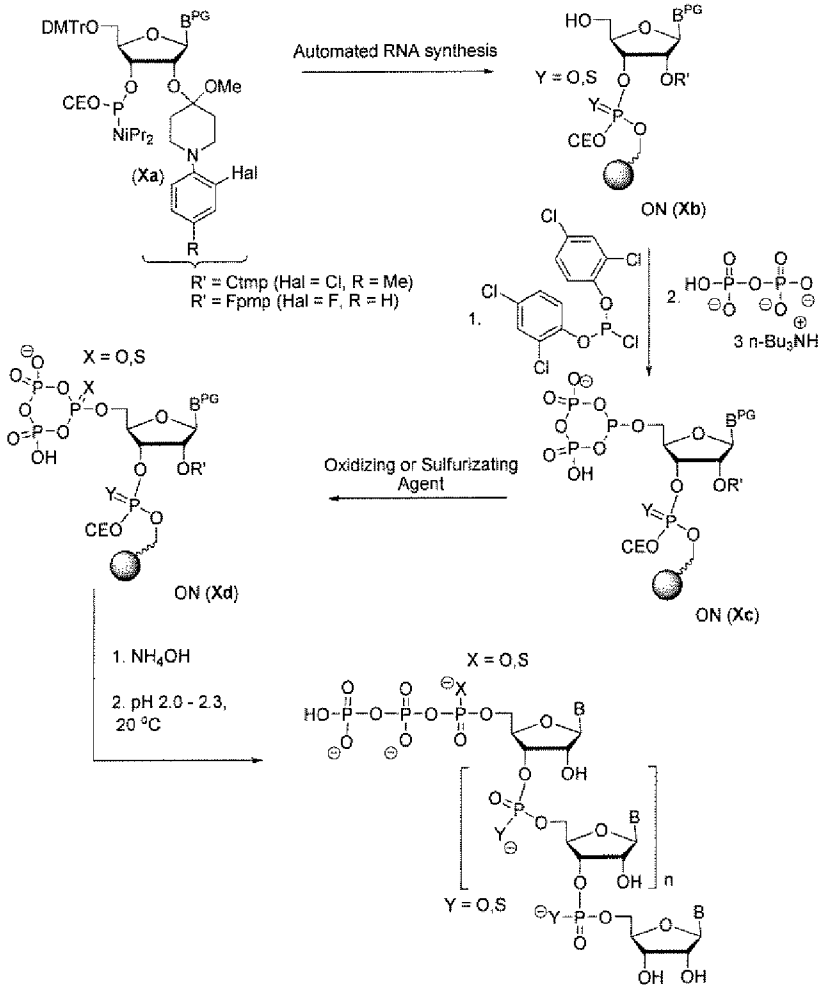


Scheme 13: The 2' Ctmp/Ftmp protected phosphoramidites (104a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 104b), as previously discussed for scheme 4. It is then involved in a phosphitylation reaction using excess O,O- bis 2,4-dichloro phosphorochloridite in solution in dry pyridine. After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (104c) is oxidized, sulfurized or reacted with a suitable boron complex,

using standard reagents. Previously described hydrolysis and deblocking steps of (104d) afford the target RNATP, RNA(αS)TP or RNA(αBH$_3$)TP.

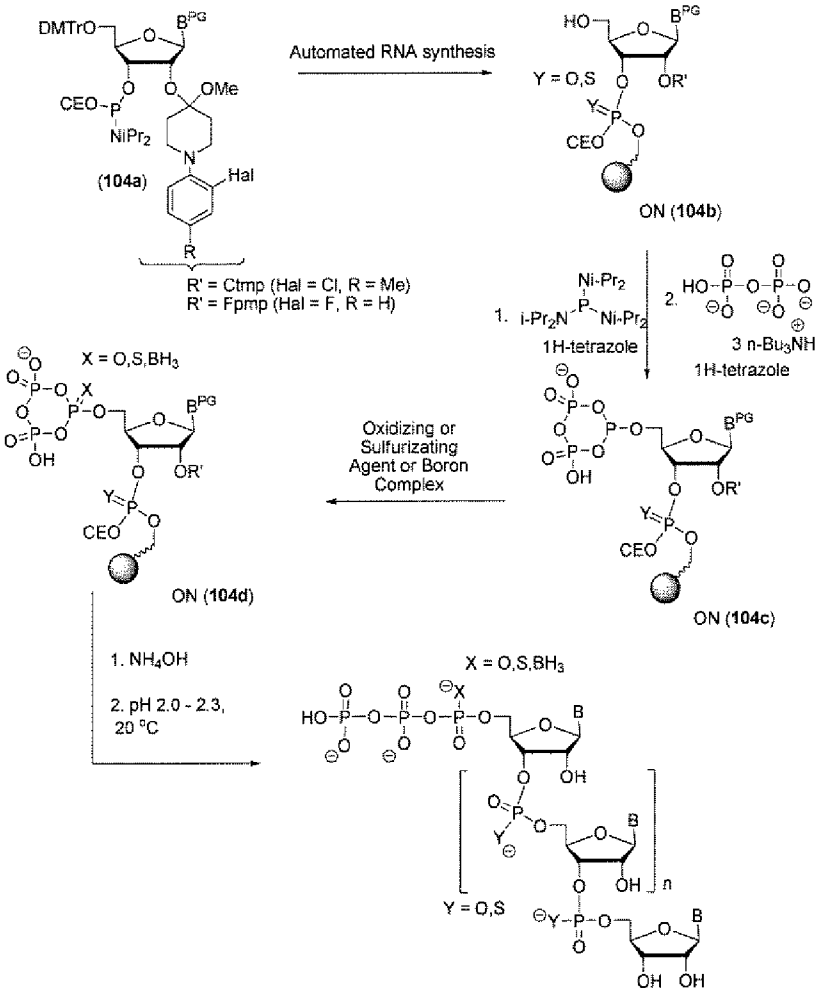
Schemes 14-17: In a general way, oligonucleotide 5' phosphorodiamidites can react, in the presence of tetrazole, with tributylammonium pyrophosphate, affording the previsouly described cyclic intermediate (c), (Shaw et al., 2003, *Org. Lett.*, 5, 2401; Fischer et al. 2002, *J. Med.Chem.*, 45, 5384). After the oxidizing/sulfurization or borane complexation step, followed by deprotection and support cleavage steps, the target RNATP or modified analog is obtained in similar fashion. Some examples exhibiting the various 2' protecting groups are depicted in schemes 9-12 and will be briefly described hereafter.

Scheme 14



Scheme 14: The 2' TBDMS protected 5' DMTr 3' phosphoramidites (105a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 105b), as previously discussed for scheme 5. It is then involved in a phosphitylation reaction using excess chloro-N,N-tetraisopropyl-phosphoro bis amidite in solution in dry pyridine. After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (105c) is oxidized, sulfurized or reacted with a suitable boron complex, using standard reagents. The corresponding cyclic TP (105d) is then hydrolyzed and deblocked as previsouly described, affording the target RNATP, RNA(αS)TP or RNA(αBH_3)TP

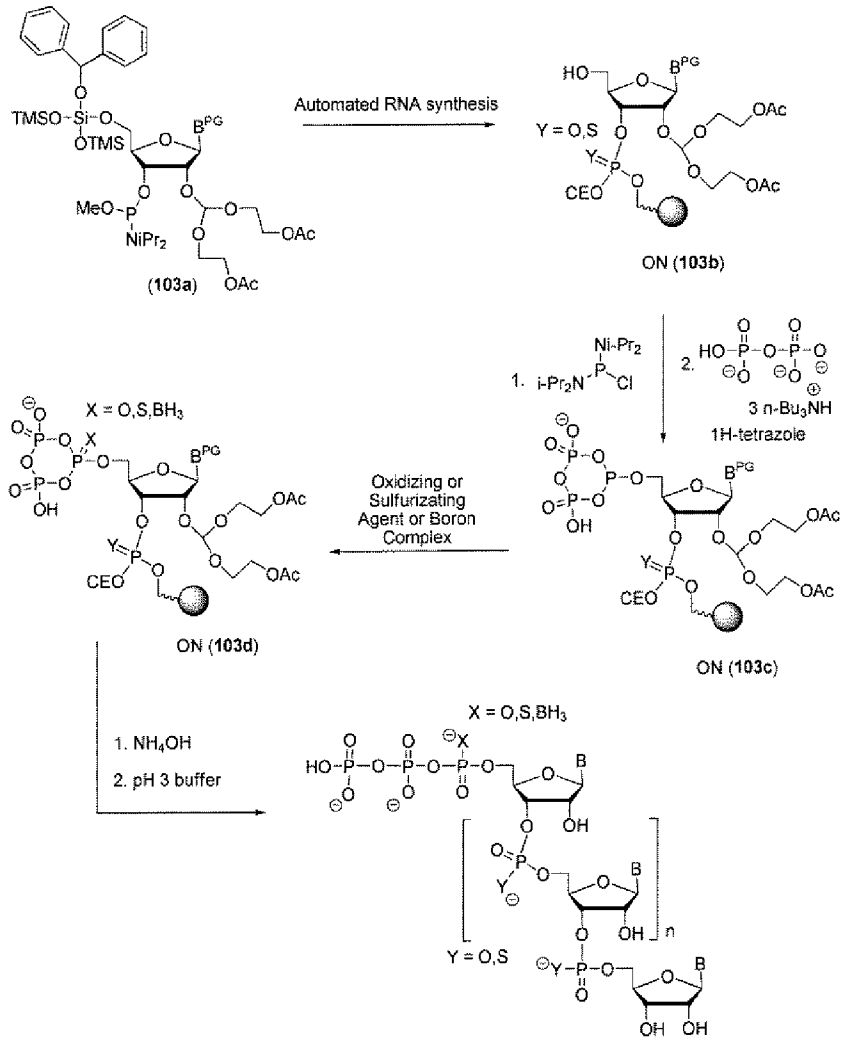
Scheme 15



Scheme 15: The 2' Ctmp/Ftmp protected phosphoramidites (104a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 104b), as previously discussed for scheme 4. It is then involved in a phosphitylation reaction using excess N,N,N',N',N'',N''-hexaaisopropyl-phosphoro tris amidite in solution of dry ACN containing tetrazole solution. After shaking the solid support with the 0.5 M solution of phosphitylation reagent and tetrazole at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (104c) is oxidized, sulfurized or reacted with a suitable boron complex, using standard reagents. Previously described

hydrolysis and deblocking steps of (104d) afford the target RNATP, RNA(αS)TP or RNA(αBH_3)TP.

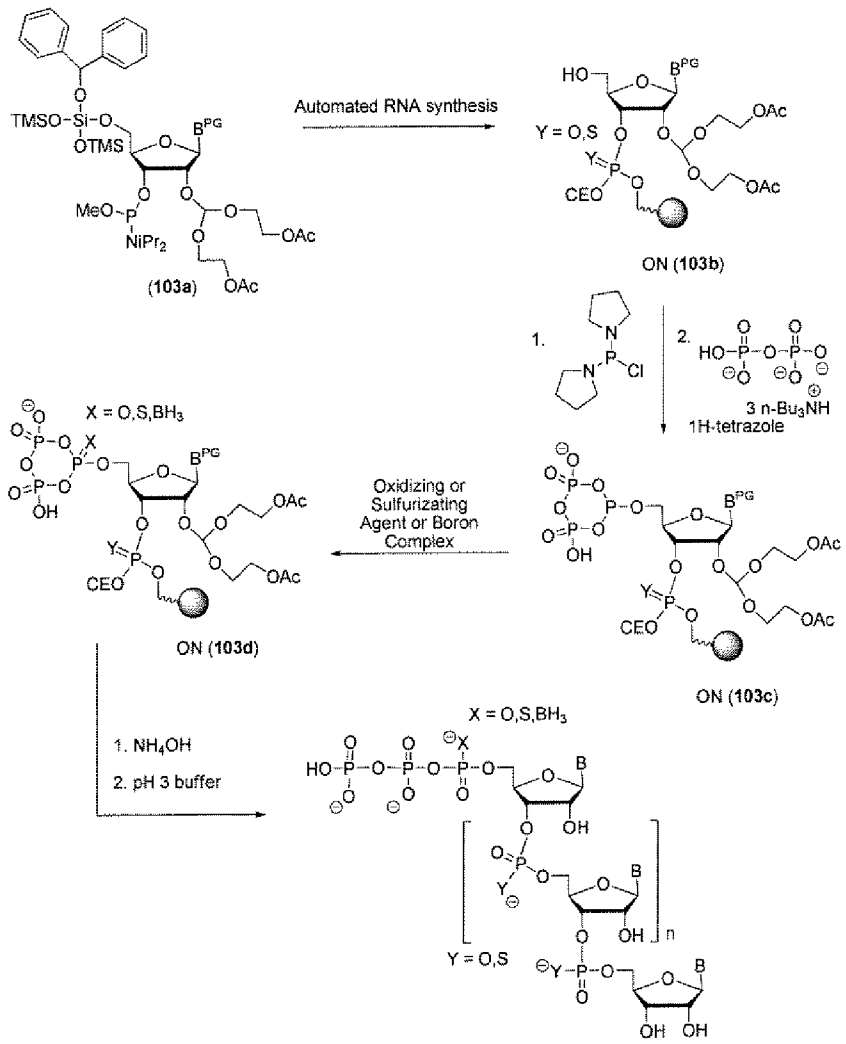
Scheme 16



Scheme 16: The 2' ACE protected phosphoramidites (103a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 103b), as previously discussed for scheme 3. It is then involved in a phosphitylation reaction using excess chloro-N,N,N',N'-tetraisopropyl-phosphoro bis amidite in solution in dry pyridine. After shaking the solid support with the 0.5 M solution of phosphitylation reagent at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported

oligonucleotide and shaken for 24 h. After washing off the solution, the resulting oligonucleotide (103c) is oxidized, sulfurized or reacted with a suitable boron complex, using standard reagents. Previously described hydrolysis and deblocking steps of (103d) affords the target RNATP, RNA(αS)TP or RNA(αBH$_3$)TP.

Scheme 17

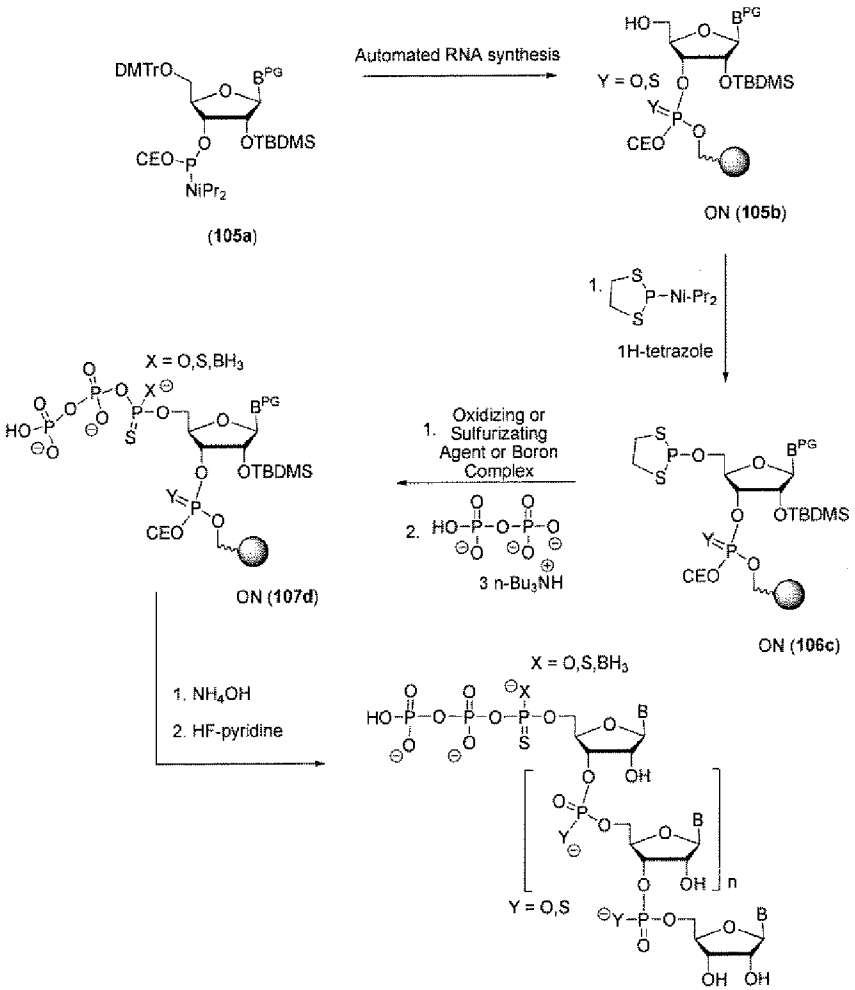


Scheme 17: The 2' ACE protected phosphoramidites (103a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 103b), as previously discussed for scheme 3. It is then involved in a phosphitylation reaction using excess chloro-phosphoro bis-pyrolo- amidite in solution in

dry pyridine. Previously described steps for scheme 11 afford the target RNATP, RNA(αS)TP or RNA(αBH$_3$)TP.

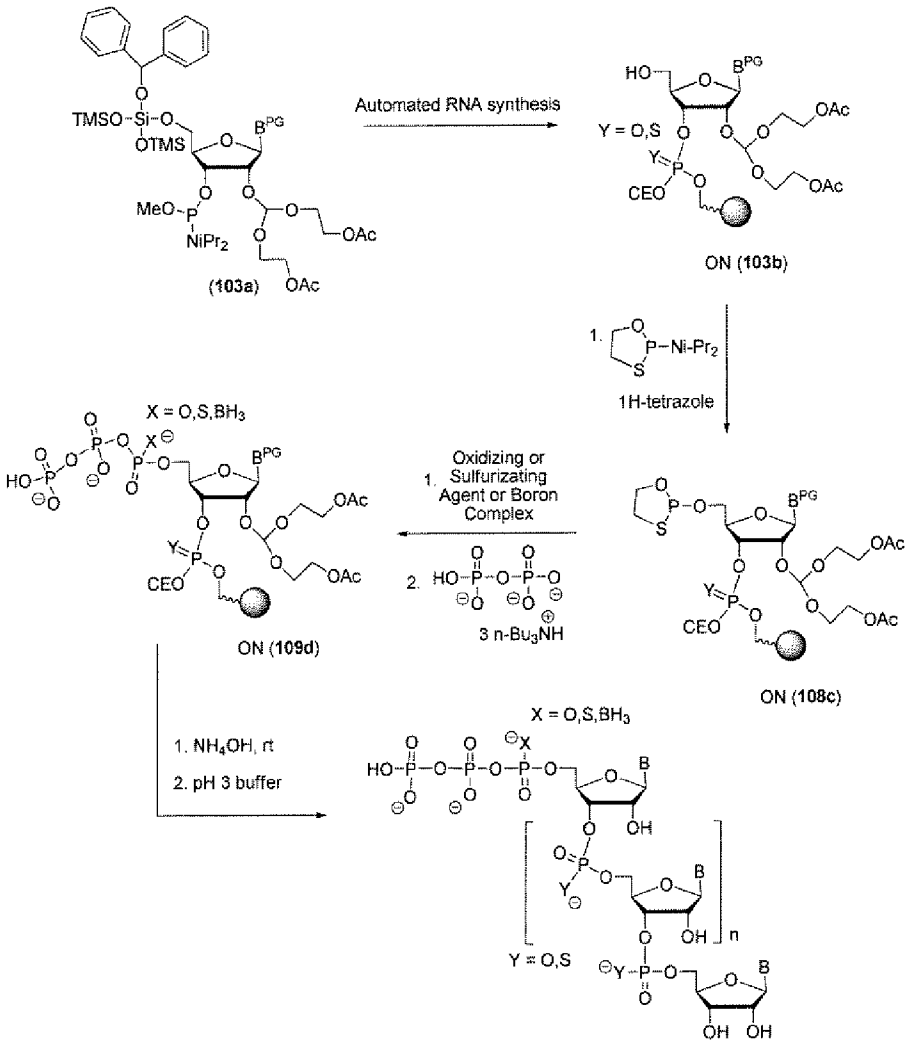
Schemes 17-19: Oligonucleotide 5' (2-thio, 2-oxo or 2-borano) oxothia or dithiaphospho-2-lanes can react with inorganic pyrophosphate in the presence of strong base catalyst DBU to give the corresponding RNATP, RNA(αS)TP, RNA(αS$_2$)TP, RNA(αBH$_3$)TP or RNA(αS,BH$_3$)TP (Okruszek et al., 1994, *J. Med. Chem.*, 37, 3850). This strategy of introduction of the modified TP moiety can be used with variation of the 2' protecting groups in a similar manner as previsouly described. Some examples are described hereafter.

Scheme 18



Scheme 18: The 2' ACE protected phosphoramidites (103a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 103b), as previously discussed for scheme 3. It is then involved in a phosphitylation reaction using excess chloro-phosphoro bis-pyrolo- amidite in solution in dry pyridine. Previously described steps for scheme 11 afford the target RNATP, RNA(αS)TP or RNA(αBH$_3$)TP.

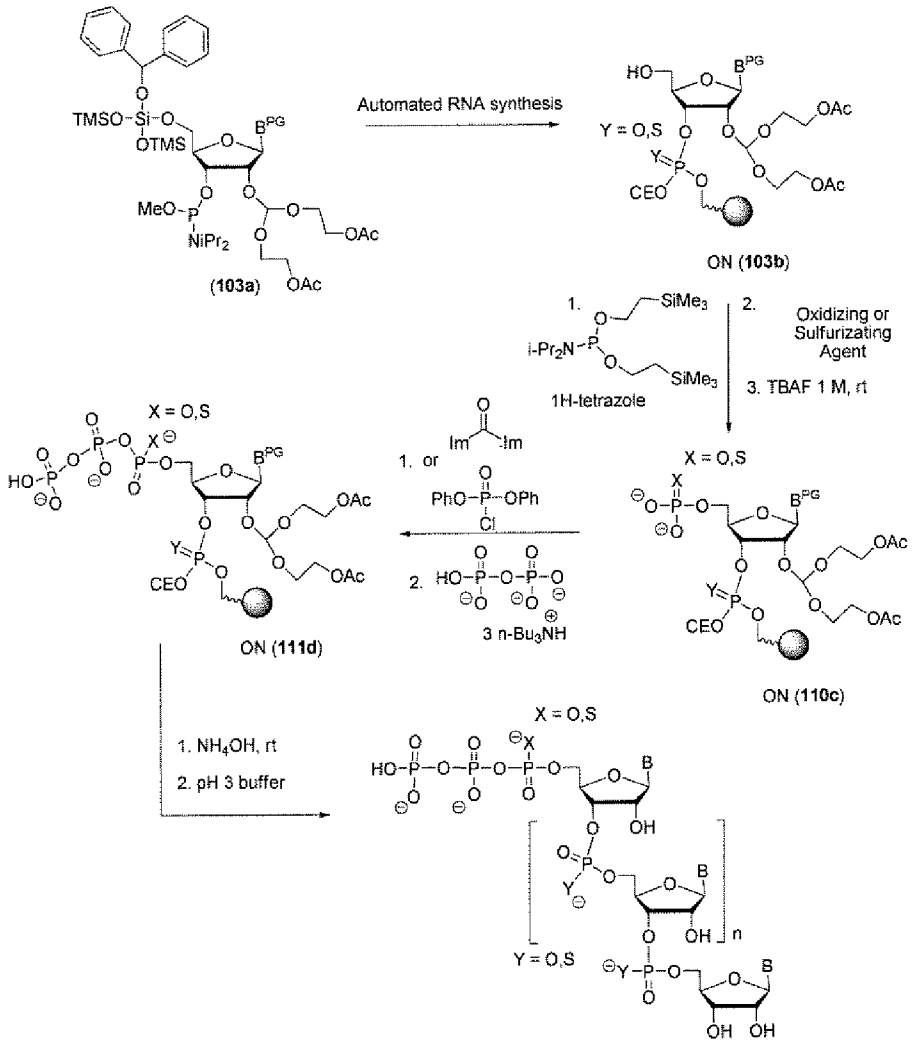
Scheme 19



Scheme 19: The 2' ACE protected 5' DMTr 3' phosphoramidites (103a) and standard solid supported RNA synthesis conditions are used for the construction of the 5' OH oligonucleotide (compound 103b), as previously discussed for scheme 5. It is then involved in a phosphitylation reaction using excess di-isopropyl oxothiaphospho-2-lane

amidite in solution in dry acetonitrile. After shaking the solid support with the 0.5 M solution of phosphitylation reagent, premixed with 0.25 M solution of tetrazole in dry ACN at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile, affording the oligonucleotide 5' oxothiaphospholane intermediate (108c), which is further oxidized, sulfurized or reacted with a suitable boron complex, using standard reagents. 0.5 M solution of tributylammonium pyrophosphate in DMF is then introduced to the solid supported oligonucleotide, along with catalytic amount of DBU and shaken for 24 h, providing opening of the dithiaphospholane cycle and elimination of the pending thioethyl group, affording the alpha thio/dithio/thioborano triphosphate intermediate (109d). This oligonucleotide TP is then hydrolyzed and deblocked as previsouly described for scheme 3, affording the target RNATP, RNA(αS)TP, or RNA(αBH_3)TP.

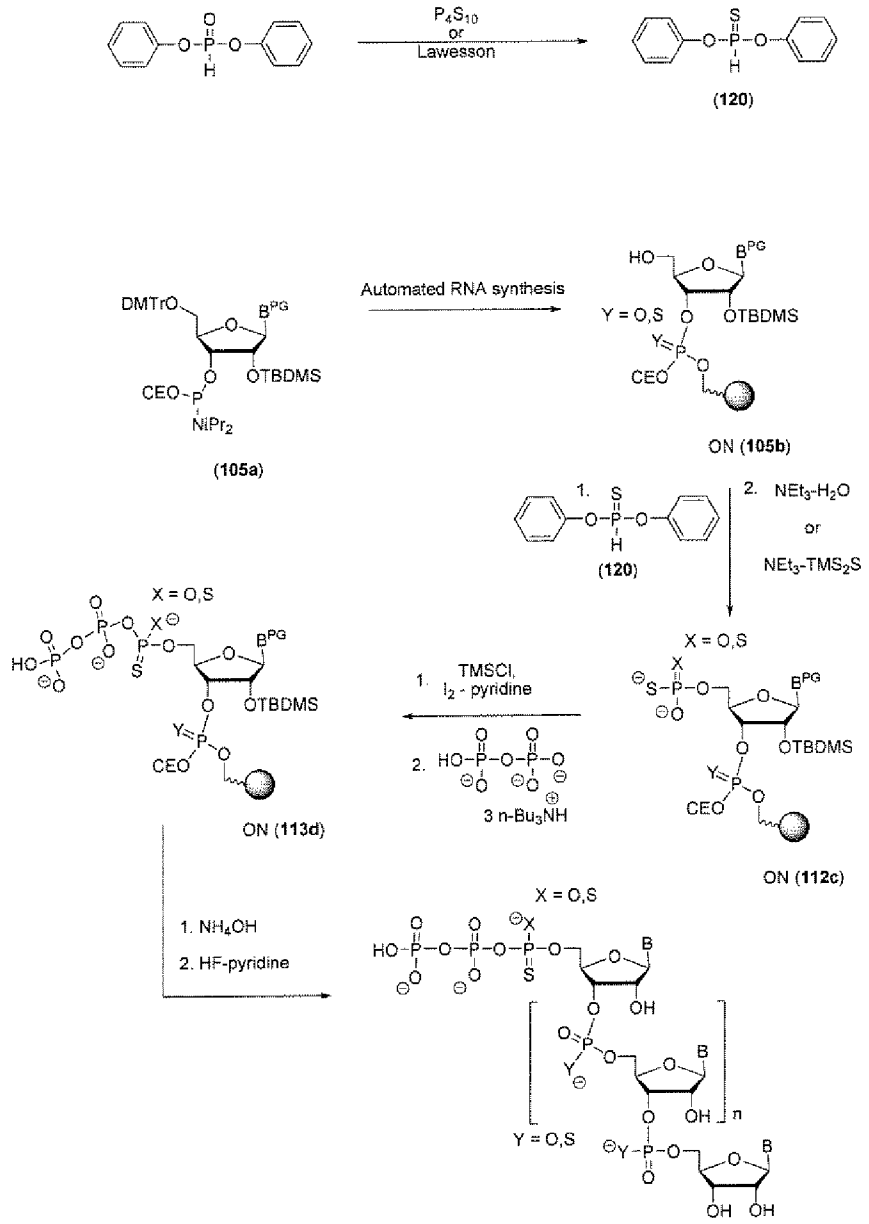
Scheme 20



Scheme 20: Nucleoside 5' monophospahtes are well known intermediates used in several efficient methods for the synthesis of the corresponding NTPs (Burgess and Cook, 2000, *Chem. Rev.*, 100, 2047). As a similar approach, the 5' monophosphate of a solid supported, conveniently protected oligonucleotide can be used in a similar fashion. O,O-Bis silyl protected 5' phosphotriester oligoucleotide can be used for the preparation of such intermediate. The 2' ACE strategy seems the most suitable for such synthesis, as the 5' phosphotriester can be deprotected employing the condtions used for each 5' step deprotection. The 2' ACE protected 5' DMTr 3' phosphoramidites (103a) and standard solid supported RNA synthesis conditions can be used for the construction of the 5' OH oligonucleotide (compound 103b), as previously discussed for scheme 5. It is then

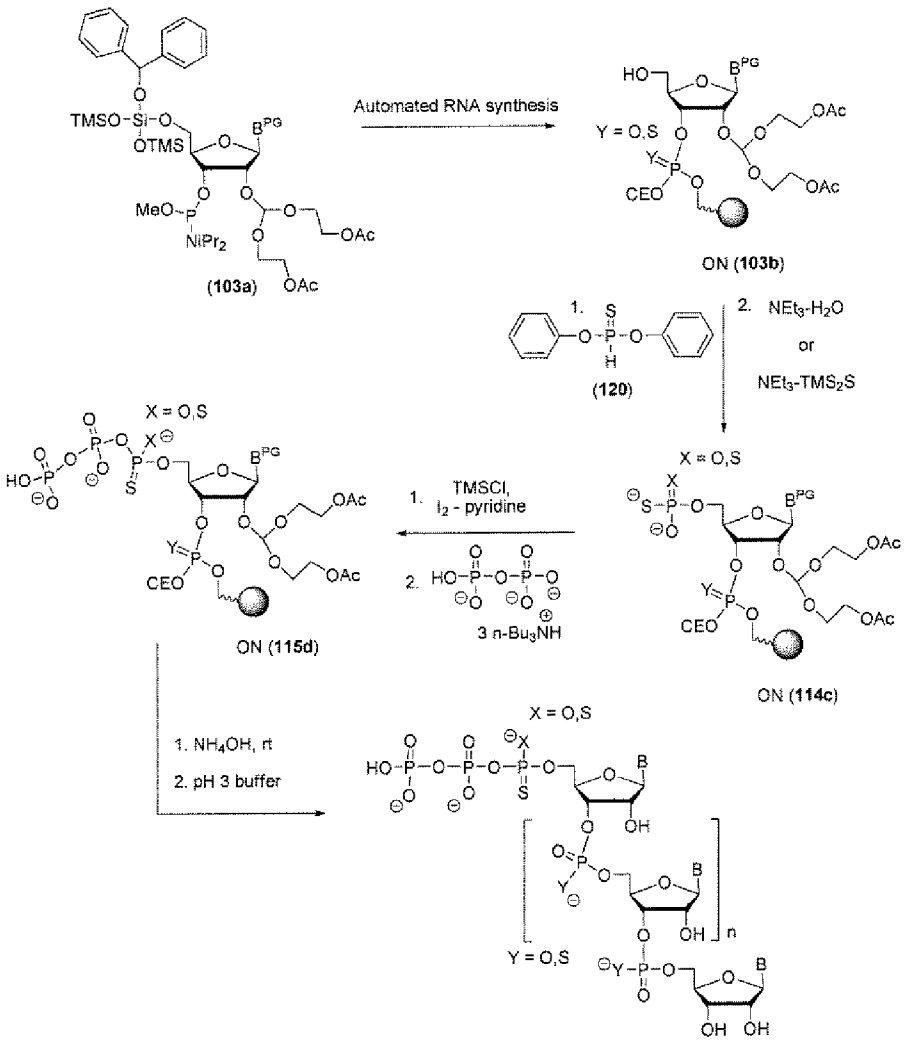
involved in a phosphitylation reaction using excess O,O-bis-trimethylsilylethyl-N,N-di-isopropylphosphoramidite in solution in dry acetonitrile. After shaking the solid support with the 0.5 M solution of phosphitylation reagent, premixed with 0.25 M solution of tetrazole in dry ACN at room temperature for 30 min, the solution is washed off and the solid support is well rinsed with dry acetonitrile, then further oxidized or sulfurized using standard reagents. Deprotection of the two TSE protectiong groups is performed using 1 M TBAF. The resulting 5' monophosphate oligonucleotide (110c) is first activated using either CDI or DPPC, and then a 0.5 M solution of tributylammonium pyrophosphate in DMF is introduced to the solid supported oligonucleotide and shaken for 24 h, affording the corresponding triphosphate intermediate (111d). This oligonucleotide TP is then hydrolyzed and deblocked ,as previsouly described for scheme 3, affording the target RNATP or RNA(αS)TP.

Scheme 21


P4S10
or
Lawesson
(120)
DMTrO
BPG
OTBDMS
CEO
NiPr2
(105a)
Automated RNA synthesis
HO
Y = O,S
ON (105b)
1.
2. NEt3-H2O
or
NEt3-TMS2S
X = O,S
1. TMSCl,
I2 - pyridine
2. HO-P-O-P-O
3 n-Bu3NH
ON (113d)
ON (112c)
1. NH4OH
2. HF-pyridine
B
OH
n
Y = O,S
OH OH

5

Scheme 22

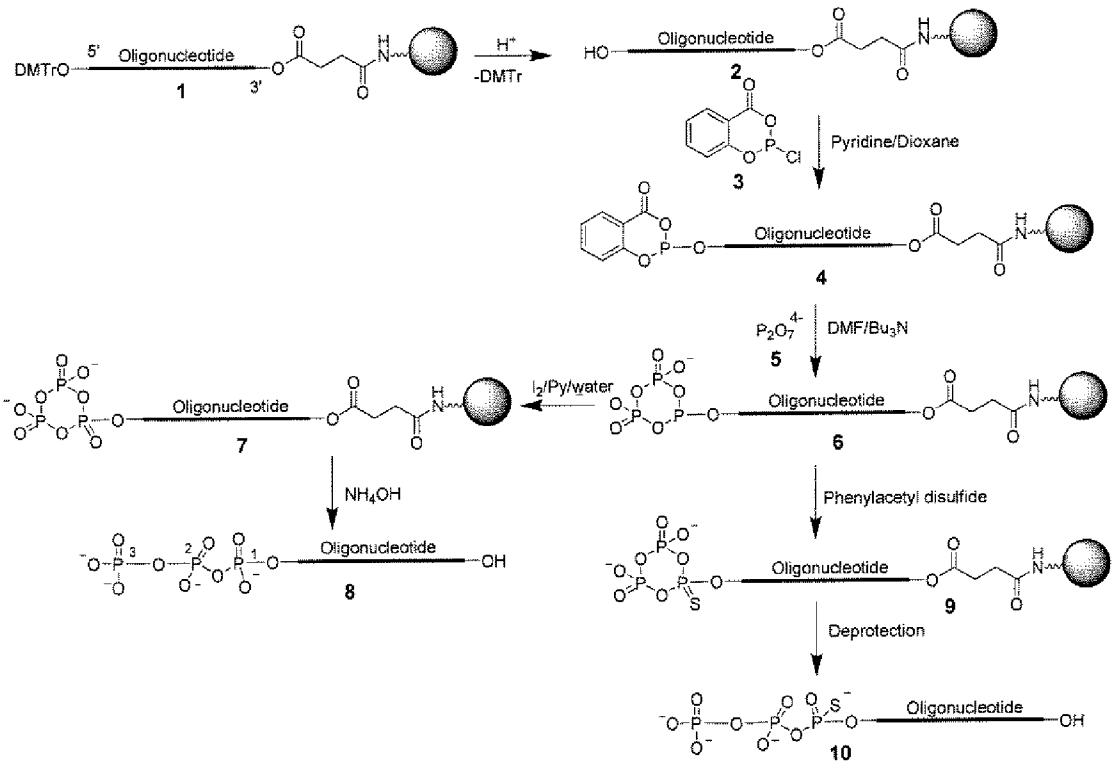


Schemes 21 and 22: Another possible path towards RNA(αS)TP and RNA(αS$_2$)TP involves the use of 5' H-phosphonothioate or phosphonodithioate oligonucleotides (112c and 114c, schemes 16 and 17, X = O, and X = S, respectively). The latter can be easily prepared after reacting the 5' OH solid supported oligonucleotide (compounds b) with diphenylthiophosphite (120, scheme 16), prepared from commercially available diphenylphosphite as previously reported (Tashma et al., 1969, *J. Org. Chem.*, 48, 3966). In such manner, 105b (scheme 16) and 103b (scheme 17) are reacted with 0.5 M solution

of 120 in dry pyridine for 1h, followed by subsequent hydrolysis (H_2O-NEt_3) or sulfolisys (TMS_2S-NEt_3), affording the corresponding compounds 112c and 114c (schemes 16 and 17, X = O, and X = S, respectively). The latter are further oxidized by iodine in the presence of a silylating agent, containing pyridine, (Peterson et al. 2008, *Org. Lett.*, 10, 1703) which allows the formation of the corresponding pyridinium thiophosphoramidate, which is reacted with tributylammonium pyrophosphate in order to provide thiotriphosphates (X = O) 113d (scheme 16) and 115d (scheme 17) or the corresponding dithiotriphosphates (X = S). After deprotection and cleavage from solid support using the conditions previously described for schemes 5 and 3, the target RNA(αS)TP and RNA(αS_2)TP are obtained.

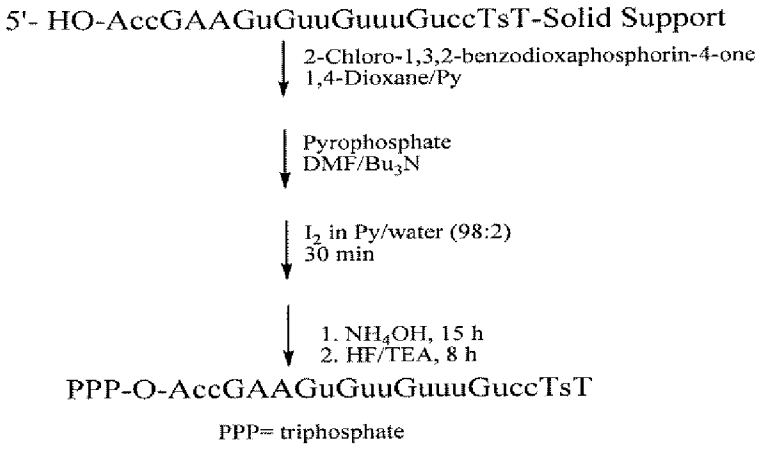
Synthesis of RNA 5'-Triphosphate:

Scheme 18[a]



[a] Synthesis of RNA 5'-triphosphate

Protocol for 5'-triphosphate synthesis



To RNA support (CPG, 200 mg) in a peptide synthesis vessel was added pyridine (0.5 ml) followed by a solution of 2-chloro-1, 3, 2-benzodioxaphosphorin-4-one (40 mg) in anhydrous 1, 4-dioxane (2 ml) under an argon atmosphere. The reaction vessel was placed on an analog shaker for 5 h. Then excess reagent was removed by applying a positive pressure of argon. The support was washed with anhydrous 1, 4-dioxane (10ml) and a solution of tributylammonium pyrophosphate (450 mg) in dry DMF (2 ml) and tributylamine (0.5 ml) were added simultaneously. After agitating the reaction vessel on an analog shaker for 22 h, excess reagent was removed by applying positive pressure of argon. To the support 1% iodine solution in pyridine and water (98:2) was added. After thirty minutes excess reagent was removed by applying positive pressure of argon. The support was washed with cold 1M TEAB buffer followed by acetone. The support was allowed to dry and transferred the support to a 20 ml vial. The support was suspended in a mixture of ethanol (1.8 ml) and ammonium hydroxide (5 ml). After agitating at room temperature for 15 h, the solution was filtered and the support was washed with 12 ml of DMSO. The solution containing RNA-triphosphate was cooled at -20C for 15 min and a cold solution of HF/TEA (7 ml) was added. The reaction flask was agitated on an analog shaker at room temperature for 8 h. A small sample of the reaction mixture was diluted with water (4 times) and analyzed by analytical HPLC using Dionex DNAPac PA-100 column. HPLC analysis of the reaction mixture clearly showed the formation of RNA-triphosphate.

The reaction mixture was diluted to 100 ml with water and the product was purified using anion exchange column chromatography. The product was eluted using a gradient of 25 mMTris (pH 8.0) to 1M ammonium chloride containing 25 mM Tris (pH 8.0) buffer. Appropriate fractions containing the product were pooled and desalted using reverse phase column chromatography.

The identity of the RNA 5'-triphosphate is established by Ion-exchange HPLC analysis and by LC-MS.

	Sequence 5' to 3'	Calc. MW	Found MW
1	HO-AccGAAGuGuuGuuuGuccTsT	6794.34	6792.88 (M-H)-
2	PPP-O-AccGAAGuGuuGuuuGuccTsT	7034.25	7033.13 (M-H)-

The lower case letters refer to 2'-OMe nucleotides.

In Figure 1, and ion-exchange HPLC analysis of purified RNA-Triphosphate is depicted.

In Figure 2, an LC-MS Analysis of RNA 5'-Triphosphate is depicted. The following data is relevant to the Figure 2 graphs:

Component	Molecular Weight	Absolute Abundance	Relative Abundance
A	7033.13	9434	100.00
B	1747.61	4119	43.66
C	2357.51	2393	25.37
D	1767.71	1707	18.09
E	2358.26	1398	14.82

Synthesis of 2'-OMe RNA Triphosphate:

2'O-Methyl RNA Triphosphate was prepared in a similar manner as described for the synthesis of RNA-triphosphate. This does not require HF/TEA treatment since it does not contain any silyl groups on the 2'-hydroxyl functions.

Synthesis of RNA Thiotriphosphate:

RNA-thiotriphosphates are prepared in a similar manner as described above. However, iodine solution is substituted with a solution of phenylacetyl disulfide in 2,6-lutidine.

Methods for identifying iRNA agents with ability to inhibit or stimulate the immune system.

Modulation of the immune system can be measured for example by (i) measurement of either the mRNA or protein expression levels of a component (*e.g.*, a growth factor, cytokine, or interleukin) of the immune system, *e.g.*, in a cell or in an animal, (ii) measurement of the mRNA or protein levels of a protein factor activated by a component of the immune system (for example, NFKB), *e.g.*, in a cell or in an animal, (iii) measurement of cell proliferation, *e.g.*, in a tissue explant or a tissue of an animal.

Evaluation of the iRNA agent can include incubating the modified strand (with or without its complement, but preferably annealed to its complement) with a biological system, *e.g.*, a sample (*e.g*, a cell culture). The biological sample can be capable of expressing a component of the immune system. This allows identification of an iRNA agent that has an effect on the component. In one embodiment, the step of evaluating whether the iRNA agent modulates, *e.g*, stimulates or inhibits, an immune response includes evaluating expression of one or more growth factors, such as a cytokine or interleukin, or cell surface receptor protein, in a cell free, cell-based, or animal assay. Protein levels can be assayed, for example, by Western blot techniques, flow cytometry, or reporter gene expression (*e.g.*, expression of a fluorescent reporter protein, such as green fluorescent protein (GFP)). The levels of mRNA of the protein of interest can be measured by Northern blot techniques, RNAse Protection Assays, or Quality Control-PCR (QC-PCR) (including quantitative reverse transcription coupled PCR (RT-PCR))

and analogous methods known in the art. RNA and/or protein levels resulting from target gene expression can be measured at regular time intervals following introduction of the test iRNA agent, and the levels are compared to those following introduction of a control iRNA agent into cells.

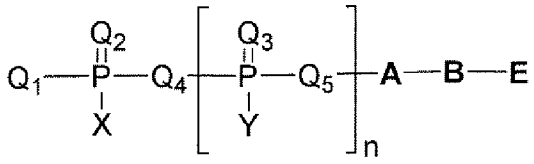
In one embodiment, the step of testing whether the modified iRNA agent modulates, *e.g.*, stimulates, an immune response includes assaying for an interaction between the iRNA agent and a protein component of the immune system, *e.g.*, a growth factor, such as a cytokine or interleukin, or a cell surface receptor protein. Exemplary assay methods include coimmunoprecipitation assays, bead-based co-isolation methods, nucleic acid footprint assays and colocalization experiments such as those facilitated by immunocytochemistry techniques.

Cell proliferaton can be monitored by following the uptake of [^{3}H]thymidine or of a fluorescent dye. Cells were plated in a 96-well tissue culture plate and then incubated with the iRNA agent. For radiometric analysis, [^{3}H]thymidine is added and incubation is continued. The cells are subsequently processed on a multichannel automated cell harvester (Cambridge Technology, Cambridge, MA) and counted in a liquid scintillation beta counter (Beckman Coulter). For fluorescence-based analysis, a commercially available assay, like the LIVE/DEAD Viability/Cytotoxicity assay from Molecular Probes can be used. The kit identifies live versus dead cells on the basis of membrane integrity and esterase activity. This kit can be used in microscopy, flow cytometry or microplate assays.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Other embodiments are in the claims.

WHAT IS CLAIMED IS:

1. An oligonucleotide of formula I, or pharmaceutically acceptable salts or prodrugs thereof:



(I)

wherein:

Q_2 and Q_3 are each, independently NH, O or S;

X and Y are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

Q_4 and Q_5 are each independently O, CH_2, CH(Me), $C(Me)_2$, CHF, CF_2, NH, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, or SR^1;

n is 0, 1, 2, 3, 4 or 5; wherein each repeating unit can be the same or different;

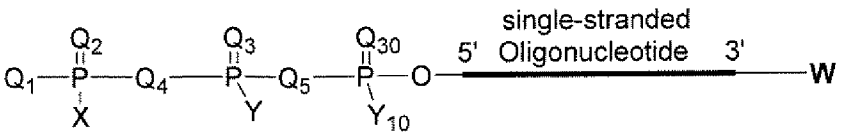
A is absent or selected from the group consisting of single-stranded oligonucleotide and double-stranded oligonucleotide, each of which may be chemically modified;

B is absent or a linker/spacer; and

E is a single-stranded oligonucleotide or a double-stranded oligonucleotide, each of which may be chemically modified and/or conjugated with a ligand;

with the proviso that when A and B are both absent and n is 0, 1 or 3, then Q_1, Q_2, Q_3, Q_4, Q_5, X and Y cannot all be oxygen.

2. The oligonucleotide of claim 1, represented by formula (II) or a pharmaceutically acceptable salt or prodrug thereof:



(II)

Q_2 , Q_3 and Q_{30} are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

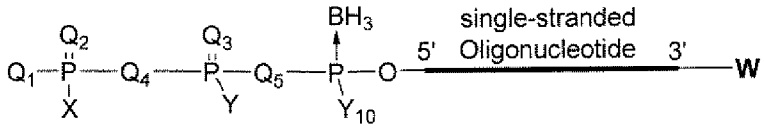
Q_4 and Q_5 are each independently O, CH_2, CH(Me), $C(Me)_2$, CHF, CF_2, NH, NR_1 or S;

Q_1 is OH, O^-, OR^1, S^-, SH, or SR^1; and

W is H, OH or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand;

provided that when W is OH, then Q_1, Q_2, Q_3, Q_4, Q_5, Q_{30}, X, Y and Y_{10} cannot all be oxygen.

3. The oligonucleotide of claim 1, represented by formula (III) or a pharmaceutically acceptable salt or prodrug thereof:



(III)

Q_2 and Q_3 are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

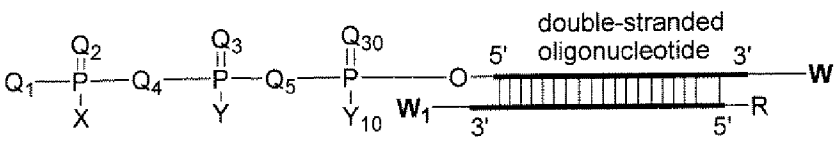
R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

Q_4 and Q_5 are each independently O, CH_2, CH(Me), $C(Me)_2$, CHF, CF_2, NH, NR_1 or S;

Q_1 is OH, O^-, OR^1, S^-, SH, or SR^1; and

W is each independently H, OH or -G-L; where G is selected from the group consisting of -CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand.

4. The oligonucleotide of claim 1, represented by formula (IV) or a pharmaceutically acceptable salt or prodrug thereof:



(IV)

Q_2 , Q_3 and Q_{30} are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

Q_4 and Q_5 are each independently O, CH_2, CH(Me), $C(Me)_2$, CHF, CF_2, NR^1, or S;

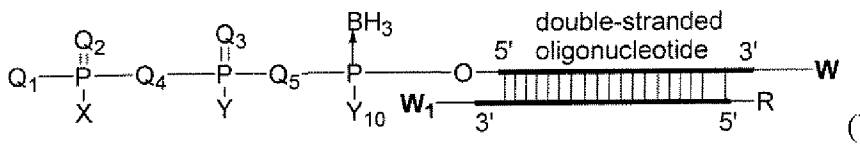
Q_1 is OH, O^-, OR^1, S^-, SH, or SR^1;

R is H, phosphate or phosphorothioate;

W and W_1 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand;

provided that when W is OH, then Q_1, Q_2, Q_3, Q_4, Q_5, Q_{30}, X, Y and Y_{10} cannot all be oxygen.

5. The oligonucleotide of claim 1, represented by formula (V) or a pharmaceutically acceptable salt or prodrug thereof:



(V)

Q_2 and Q_3 are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

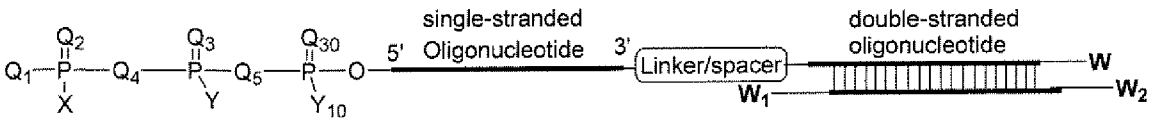
Q_4 and Q_5 are each independently O, CH_2, CH(Me), $C(Me)_2$, CHF, CF_2, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, or SR^1;

R is H, phosphate or phosphorothioate;

W and W_1 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand.

6. The oligonucleotide of claim 1, represented by formula (VI) or a pharmaceutically acceptable salt or prodrug thereof:



(VI)

Q_2 , Q_3 and Q_{30} are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

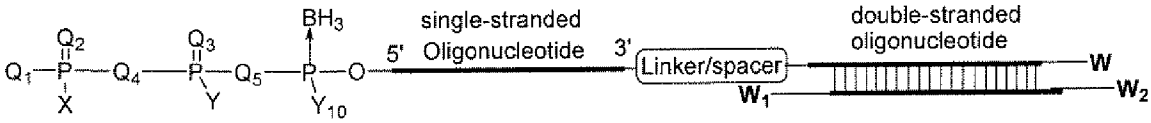
Q_4 and Q_5 are each independently O, CH_2, CH(Me), $C(Me)_2$, CHF, CF_2, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, or SR^1;

W, W_1 and W_2 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand; and

Linker/spacer is selected from the group consisting of phosphate, phosphorothioate, phosphorodithioate, alkylphosphonate, amide, ester, disulfide, thioether, oxime, hydrazone, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, and heteroaryl.

7. The oligonucleotide of claim 1, represented by formula (VII) or a pharmaceutically acceptable salt or prodrug thereof:



(VII)

Q_2 and Q_3 are each, independently NH, O or S;

X and Y and Y_{10} are each, independently, OH, O^-, OR^1, O^-, SH, S^-, Se, BH_3, BH_3^-, H, $N(R^2)_2$, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^1 is independently alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

R^2 is independently hydrogen, alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

Q_4 and Q_5 are each independently O, CH_2, CH(Me), $C(Me)_2$, CHF, CF_2, NR^1, or S;

Q_1 is OH, O^-, OR^1, S^-, SH, or SR^1;

W, W_1 and W_2 are each independently H, OH, phosphate, phosphorothioate or -G-L; where G is selected from the group consisting of –CONH-, -NHCO-, -S-S-, -OC(O)NH-, -NHC(O)O-, -NHC(O)NH-, acetal, ketal, -O-N=C-, -NH-N=C-, -S-, -O-, pyrolidine, morpholine, piperazine and thiazolidine; and where L is a ligand; and

Linker/spacer is selected from the group consisting of phosphate, phosphorothioate, phosphorodithioate, alkylphosphonate, amide, ester, disulfide, thioether, oxime, hydrazone, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, and heteroaryl.

8. The oligonucleotide of any of claims 1-7, wherein the single- or double-stranded oligonucleotide comprises at least one modified nucleotide.

9. The oligonucleotide of claim 8, wherein at least one of said modified nucleotides is selected from the group consisting of a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group.

10. The oligonucleotide of claim 8, wherein at least one of said modified nucleotides is selected from the group of a 2'-fluoro nucleotide, a 2'-*O*-alkyl nucleotide, a 2'-*O*-alkoxyalkyl nucleotide, a 2'-*O*-allyl nucleotide, a 2' *O*-propyl nucleotide, a 2'-*O*-methylated nucleotide (2'-*O*-Me), a 2'-deoxy nucleotide, a 2'-deoxyfluoro nucleotide, a 2'-*O*-methoxyethyl nucleotide (2'-*O*-MOE), a 2'-*O*-N-MeAcetamide nucleotide (2'-*O*-NMA), a 2'-*O*-dimethylaminoethyloxyethyl nucleotide (2'-*O*- DMAEOE), a 2'-aminopropyl, a 2'-hydroxy, a 2'-ara-fluoro, a 3'-amidate (3'-NH in place of 3'-O), a locked oligonucleotide (LNA), an extended ethylene oligonucleotide (ENA), a hexose oligonucleotide (HNA), or a cyclohexene oligonucleotide (CeNA).

11. The oligonucleotide of any of claims 1-10, wherein the oligonucleotide is comprised in a viral vector.

12. The oligonucleotide of any of claims 1-10, wherein the oligonucleotide binds to RIG-I.

13. A composition comprising an oligonucleotide as defined in any of claims 1-12 and at least one agent selected from the group consisting of an immunostimulatory agent, an anti-viral agent, an anti-bacterial agent, an anti-tumor agent, a gene-silencing agent, an anti-tumor therapy, and combinations thereof.

14. The composition of claim 13, wherein the agent is retinoic acid, type I IFN, or a combination thereof.

15. Use of the oligonucleotide as defined in any of claims 1-12 for inducing apoptosis of tumor cells, inducing an anti-viral response, inducing an anti-bacterial response, and/or inducing an anti-tumor response in a vertebrate animal.

16. The use of claim 15, wherein the anti-viral response, the anti-bacterial response and/or the anti-tumor response comprise type I IFN production, IL-18 production, and/or IL-1β production.

17. A composition comprising an oligonucleotide as defined in any of claims 1-12 and at least one antigen for inducing an immune response against an antigen in a vertebrate animal.

18. The composition of claim 17, wherein the oligonucleotide is covalently linked to the at least one antigen.

19. Use of the oligonucleotide as defined in any of claims 1-12 for the preparation of a medicament for preventing and/or treating a disease and/or disorder selected from the group consisting of viral infection, bacterial infection, parasitic infection, tumor, multiple sclerosis, allergy, autoimmune diseases, immunosuppression, and immunodeficiency in a vertebrate animal.

20. Use of the oligonucleotide as defined in any of claims 1-12 for the preparation of a medicament for inducing apoptosis of tumor cells, inducing an anti-viral response,

inducing an anti-bacterial response, and/or inducing an anti-tumor response in a vertebrate animal.

21. The use of claim 20, wherein the anti-viral response, the anti-bacterial response, and/or the anti-tumor response comprise type I IFN production, IL-18 production, and/or IL- 1β production.

22. A pharmaceutical composition comprising a oligonucleotide as defined in any of claims 1-12.

23. A combined preparation, comprising an oligonucleotide as defined in any of claims 1-12 and at least one agent selected from the group consisting of an immunotstimulatory agent, an anti-viral agent, an anti-bacterial agent, an anti-tumor agent, and a gene silencing agent, wherein the oligonucleotide and the at least one agent are administered simultaneously, separately, or sequentially.

24. The combined preparation of claim 23, wherein the agent is retinoic acid, type I IFN, or a combination thereof.

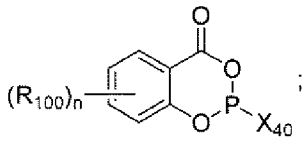
25. A pharmaceutical package, comprising the pharmaceutical composition of claim 22 or the combined preparation of claim 23 or 24 and an instruction for use.

26. Use of the oligonucleotide as defined in any of claims 1-12 for the preparation of a bacterial RNA for preventing and/or treating a disease and/or disorder selected from the group consisting of viral infection, bacterial infection, parasitic infection, tumor, multiple sclerosis, allergy, autoimmune diseases, immunosuppression, and immunodeficiency in a vertebrate animal.

27. A process of preparing oligonucleotide molecule having one or more ribonucleotides comprising a triphosphosphate or a triphosphate analog, comprising the steps of:

(a) protecting the 2'hydroxyl moiety with a fluoride labile group or a fluoride non-labile group;

(b) converting the desired terminal hydroxyl moiety to a triphosphate or triphosphate analog with a reagent selected from the group consisting of: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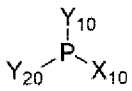
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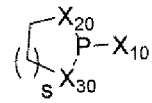
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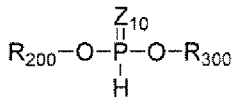
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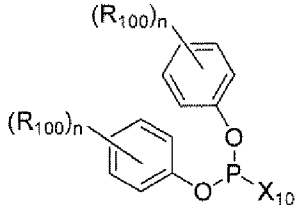
and

;

wherein:
R_{100} is independently electron withdrawing group (EWG);
R_{200} and R_{300} are earch independly haloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, cycloalkyl, subsituted cycloalkyl, heterocycle, substituted heterocyclo;
Z_{10} is O, S, Se, BH_3 or NR';
X_{40} is Cl, dialkylamine or cyclic amine;
X_{10} is Cl, O-aryl or O-substituted aryl;
Y_{10} and Y_{20} are independently O-substituted alkyl; dialkylamine or cyclic amine, wherein the nitrogen is connected to the phosphorus;
X_{20} and X_{30} is independently O, CH_2, S, NR', wherein R' is H or aliphatic;
n is 1, 2, 3, 4, or 5; and
s is 0, 1, 2 or 3;

(c) synthesizing said oligonucleotide molecule using a method selected from the group consisting of solid phase phosphoramidite, solution phase phosphoramidite, solid phase H-phosphonate, solution phase H-phosphonate, hybrid phase phosphoramidite, and hybrid phase H-phosphonate-based synthetic methods; and (d) removing the protecting group(s) and/or solid support.

28. The pharmaceutical composition of claim 22, adapted for delivery by a mode selected from the group consisting of intraocular injection, oral ingestion, enteral application, inhalation, topical application, subcutaneous injection, intramuscular injection, intraperitoneal injection, intrathecal injection, intratrachael injection, and intravenous injection.

29. A kit comprising a oligonucleotide of claim 1 in a labeled package and the label on said package indicates that said oligonucleotide can be used against at least one virus.

30. The kit of claim 30, wherein said kit is approved by a regulatory agency for use in humans.

31. An assay for indentifying an anti-viral or an antibacterial response, comprising contacting a test sample with a test agent, wherein the test agent comprises an oligonucleotide as defined in any of claims 1-12.

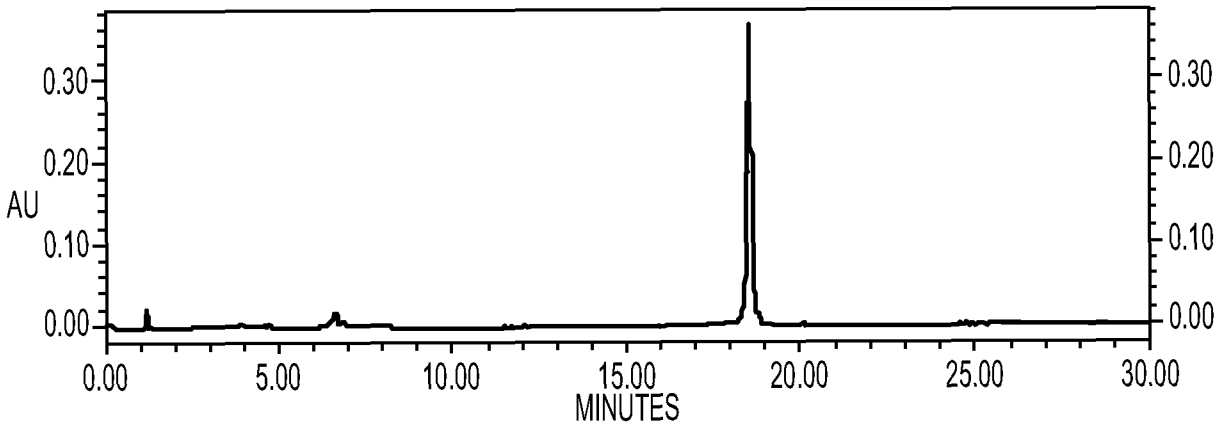
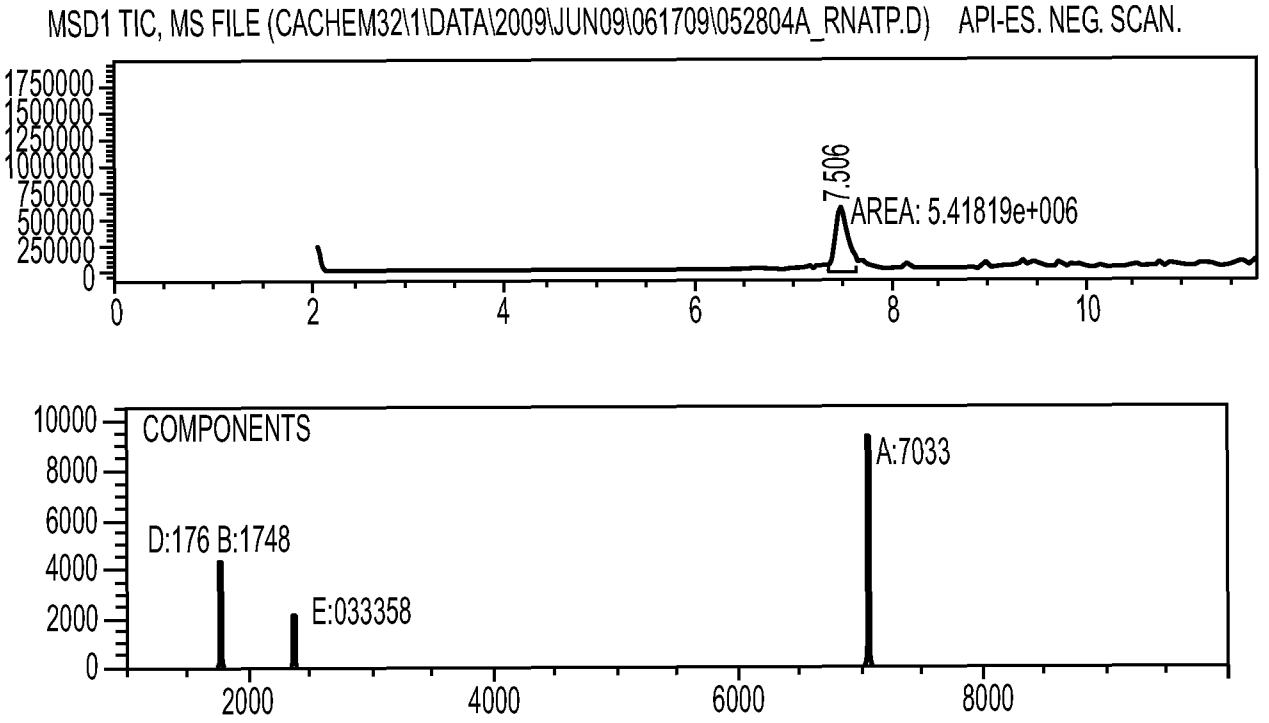

AU
0.30
0.20
0.10
0.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00
MINUTES

FIG. 1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1750000
1500000
1250000
1000000
750000
500000
250000
0
7.506
AREA: 5.41819e+006
0
2
4
6
8
10
10000
8000
6000
4000
2000
0
COMPONENTS
A:7033
D:176 B:1748
E:033358
2000
4000
6000
8000

FIG. 2