(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 January 2001 (11.01.2001)

PCT

(10) International Publication Number
WO 01/02608 A1

(51) International Patent Classification[7]: C12Q 1/68, C12P 19/34, C07H 21/00, 21/00, 21/02, 21/04, A01N 43/04

(21) International Application Number: PCT/US00/18415

(22) International Filing Date: 5 July 2000 (05.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/348,106 6 July 1999 (06.07.1999) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/02608 A1

(54) Title: OLIGONUCLEOTIDES INCORPORATING BOTH 2-AMINOADENINE AND 5-SUBSTITUTED PYRIMIDINES

(57) Abstract: This invention presents oligonucleotides incorporating both 2-aminoadenine and 5-substituted pyrimidines together with a modified internucleoside linkage. In certain preferred embodiments, the 5-substituted pyrimidine is 5-methylcytosine. The oligonucleotides produced provide stronger hybridization to their target sequences. These oligonucleotides can be useful compounds, *inter alia* for diagnostic and therapeutic applications.

OLIGONUCLEOTIDES INCORPORATING BOTH 2-AMINOADENINE AND 5-SUBSTITUTED PYRIMIDINES

FIELD OF THE INVENTION

This invention is related to oligonucleotides having non-standard bases. In particular, this invention provides oligonucleotides having a combination of 2-aminoadenine and 5-substituted pyrimidines. In preferred embodiments, the 5-substituted pyrimidine is 5-methylcytosine.

BACKGROUND OF THE INVENTION

Oligonucleotides and their analogs have been developed for various uses in molecular biology, including use as probes, primers, linkers, adapters, and gene fragments. In a number of these applications, the oligonucleotides specifically hybridize to a target nucleic acid sequence. Hybridization is the sequence specific hydrogen bonding of oligonucleotides via Watson-Crick and/or Hoogsteen base pairs to RNA or DNA. The bases of such base pairs are said to be complementary to one another.

One application utilizing an oligonucleotide's ability to hybridize to a complementary region in RNA or DNA is the diagnostic testing of materials including, for example, biological fluids, tissues, intact cells and isolated cellular components. There are numerous examples of commercially available kits using probe technologies that hybridize to a target sequence for diagnostic purposes.

Oligonucleotides are also widely used as research reagents. They are particularly useful in studies exploring the function of biological molecules, as well as in the preparation of biological molecules. For example, the use of both natural and synthetic oligonucleotides as primers in PCR reactions has given rise to an expanding commercial industry. PCR has become a mainstay of commercial and research laboratories, and applications of PCR have multiplied. For example, PCR technology now finds use in the fields of forensics, paleontology, evolutionary studies and genetic counseling. Commercialization has led to the development of kits which assist non-molecular biology-trained personnel in applying PCR.

Oligonucleotides are also used in other laboratory procedures. Several of these uses are described in common laboratory manuals such as *Molecular Cloning, A Laboratory Manual*, Second Ed., J. Sambrook, et al., Eds., Cold Spring Harbor Laboratory Press, **1989**; and *Current Protocols In Molecular Biology*, F. M. Ausubel, et al., Eds., Current Publications, **1993**. Representative uses include synthetic oligonucleotide probes, screening expression libraries with antibodies and oligonucleotides, DNA sequencing, In Vitro Amplification of DNA by the Polymerase Chain Reaction and Site-directed Mutagenesis of Cloned DNA. *See* Book 2 of *Molecular Cloning, A Laboratory Manual, supra*, and DNA-Protein Interactions and The Polymerase Chain Reaction, Vol. 2 of *Current Protocols In Molecular Biology, supra*.

Another method of use for oligonucleotides involving hybridization is the inhibition of specific gene expression, where the oligonucleotides are complementary to specific target messenger RNA (mRNA) or other sequences. This mode of action is commonly known as "antisense". The specific binding of an antisense oligonucleotide to its target mRNA or other sequence can inhibit gene expression by at least two major mechanisms. The binding of the oligonucleotide to its target may hinder protein binding for translation and/or regulation. Moreover, the oligonucleotides may act through RNase-mediated degradation of a DNA/RNA duplex. Antisense technology is used in research applications to study the functions of certain genes. Antisense oligonucleotides can also be therapeutic agents, with one antisense drug having been approved for use and several oligonucleotides currently undergoing clinical trials.

Other oligonucleotide based therapeutics include ribozymes and triplex forming oligonucleotides (TFOs). With ribozymes, a region of the oligonucleotide hybridizes to a complementary region in a target RNA. With TFOs, the oligonucleotide hybridizes to a complementary DNA strand, displacing the other DNA strand. Both of these therapeutic strategies could also benefit from increased hybridization to their targets.

It is greatly desired that oligonucleotides be able to be synthesized to have customized properties which are tailored for desired uses. Thus a number of chemical modifications have been introduced into oligonucleotides to increase their usefulness in diagnostics, as research reagents and as therapeutic entities. Such modifications include those designed to increase hybridization or binding to a target strand (i.e. increase their melting temperatures, Tm), to assist in identification of the oligonucleotide or an oligonucleotide-target complex, to increase cell penetration, to stabilize against nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotides, to provide a mode of disruption (a terminating event) once sequence-specifically bound to a target, to improve the pharmacokinetic properties of the oligonucleotide and to reduce immune stimulation.

These chemical modifications to oligonucleotides generally fall into three classes, alternative internucleoside linkages, modified sugars and non-standard bases.

Phosphorothioate oligonucleotides, which incorporate a phosphorothioate internucleoside linkage (P=S), as opposed to a phosphodiester linkage (P=O), are a very common modification and are presently being used as antisense agents in human clinical trials for various disease states, including use as antiviral, anticancer and anti-autoimmune agents.

Oligonucleotides bind in a sequence specific manner to their target, a stretch of nucleotides from either DNA or RNA, especially messenger RNA (mRNA). The base-pairing of such interactions is the well known; A-T (or A-U) and G-C base pairing. The G-C base pair provides three hydrogen bonds, while the A-T and A-U base pairs provide only two. Thus, the A-T base pair is energetically less favorable. By increasing the A-T base pair to have three hyrogen bonds, through chemical modification of these bases, it has been shown that a more stable duplex structure can be formed. This increased hybridization is expected to have wide-reaching application in molecular biology. Diagnostic probes and therapeutic agents are expected to be more effective.

The incorporation of 2-aminoadenosine (2,6-diamino-purine) and similar moieties into oligonucleotides in place of adenosine provides an additional site for hydrogen bonding to uridine or thymidine. This modification has been shown to increase the binding affinity of oligonucleotides to their target RNA sequences and, to a lesser extent, DNA sequences (Gryaznov, S., *et al., Tetrahedron Lett.* **1994**, *35*, 2489-2492).

The use of oligonucleotides containing 2-amino-adenosine has been described in Lamm, G.M., *et al.* (*Nucleic Acids Res.* **1991**, *19*, 3193-3198) and Barabino, S.M., *et al.*, (*Nucleic Acids Res.* **1992**, *20*, 4457-4464) wherein 2'-O-allyl antisense probes containing 2-aminoadenine were used to deplete U5 snRNP from cell extracts and to inhibit exon ligation. Oligonucleotides without 2-aminoadenosine were less effective.

Modifications of the 5 position of cytosines has been found to be effective in reducing immune stimulation (Boggs, R.T., *et al., Antisense Nucleic Acid Drug Dev.*, **1997**, *7*, 461-471), particularly 5-methyl and 5-halo substitutions. In addition, these modifications may be increase hybridization of oligonucleotides to target. Substitution of a single cytosine with 5-methyl cytosine was also able to stabilize an oligonucleotide by increasing its T_M by 0.5°C (Sanghvi, Y.S., *et al., Nucleic Acids Res.*, **1993**, *21*, 3197-3203).

The use of 5-methylcytosine in antisense oligonucleotides has been described in Witters, L., *et al.* (*Breast Cancer Res. Treat.*, **1999**, *53*, 41-50).

Oligonucleotides containing both 2-aminoadenosine and 5-methylcytidine have been used in limited diagnostic applications. Prosnyak, M.I., *et al.* (*Genomics*, **1994**, *21*, 490-494) used a $(CAC)_5$ sequence where all the cytidines were 5-methylcytidines and all the adenosines were 2-aminoadenosine as a DNA fingerprinting probe. PCR primers containing both these modifications were used by Lebedev, Y., *et al.* (*Genet. Anal. Biomol. Eng.*, **1996**, *13*, 15-21). Bailly, C., *et al.* (*Mol. Pharm.*, **1998**, *53*, 77-87) synthesized large DNA fragments containing these modified bases for use in DNase I footprinting studies with the antibiotic, rebeccamycin. Buttinelli, M. *et al.* (*Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 8544-8549) also use PCR to synthesize DNA fragments containing both these modifications for use in studying histone binding to DNA.

The combination of 5-methylcytosine and 2-aminoadenine has also been used in hexitol nucleic acids (Boudou, V., *et al., Nucl. Acids Res.*, **1999**, *27*, 1450-1456). The combination of base modifications and the non-standard sugar was found to be additive.

There remains a need for improved oligonucleotide compositions incorporating both 2-aminoadenine and 5-substituted pyrimidines.

SUMMARY OF THE INVENTION

In accordance with this invention there are provided oligonucleotides comprising at least one 2-aminoadenosine nucleoside, at least one 5-substituted pyrimidine nucleoside and a modified internucleoside linkage. Additional oligonucleotides are provided comprising at least one 2-aminoadenosine nucleoside, at least one 5-substituted pyrimidine nucleoside, a modified internucleoside linkage and a modified sugar residue. In preferred embodiments of the invention, the 5-substituted pyrimidine is a 5-methylcytosine. Preferred internucleoside linkages are phosphorothioate linkages. Preferred modified sugar residues are 2'-O-methoxyethyl-modified sugars (giving rise to a 2'-methoxyethoxy functionality). Other 2' alkoxy and alkoxy-alkoxy functions are also preferred. Methods are also provided for inhibiting gene expression using the oligonucleotides of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The compounds of the present invention comprise oligonucleotides incorporating both 2-aminoadenine and 5-substituted pyrimidines. In a preferred embodiment, the 5-substituted pyrimidine is 5-methylcytosine. Analogs of each of these base substitutions are comprehended by the present invention. The resulting oligonucleotides provide stronger hybridization to their target sequences and are important compounds for diagnostic, therapeutic and other applications.

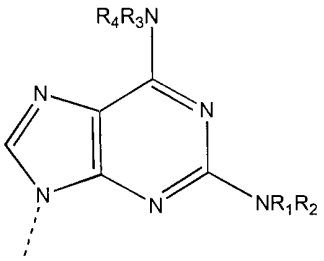
For the purposes of the present invention, 5-substituted pyrimidine is defined as a pyrimidine base with a substitution as described in Luyten, I. and Herdewijn, P. (*Eur. J. Med. Chem.*, **1998**, *33*, 515-576) at the 5 position. Included are 5-halo pyrimidines, e.g. 5-bromo, 5-chloro, 5-iodo, and 5-fluoro, pyrimidines as well as pyrimidines having hydrocarbyl groups such as alkyl or substituted alkyl, alkenyl or substituted alkenyl, alkynyl or substituted alkynyl, cycloalkyl, cycloalkenyl, cycloaralkyl, aryl, aralkyl or substituted aralkyl at the 5 position. Preferably, alkyl, alkenyl and alkynyl substituents, collectively hydrocarbyls, are groups having from 1 to about 30 carbons, with 1 to about 10 carbons being particularly preferred. Preferably, the aryl groups have from 6 to about 14 carbons, and aralkyl groups

have from 7 to about 30 carbons. The substituent groups listed above can themselves bear substituent groups such as alkoxy, hydroxyl, amine, benzyl, phenyl, nitro, thiol, thioalkoxy, halo, or alkyl, aryl, alkenyl, or alkynyl. Particularly preferred are 5-methylcytosine and 5-fluoro cytosine.

It will be appreciated by those skilled in the art that a 5-substituted pyrimidine will typically be attached to a sugar moiety. Such 5-modified pyrimidine nucleosides can be incorporated into oligonucleotides in ways well known to persons of ordinary skill in the art, especially via automated methods. Other structures such as those employing modified sugars, modified nucleotide bond and otherwise are also useful herein. 5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides can be synthesized according to published methods (Sanghvi et al., *Nucl. Acids Res.*, **1993** *21*, 3197).

For the purposes of the present invention, 2-aminoadenine is defined to include 2-aminoadenine as well as N2 substituted 2-aminoadenine analogs such as those described in US Patent 5,459,255, commonly assigned and herein incorporated by reference. Also included are N6 substituted 2-aminoadenine analogs. A number of substituent groups can be introduced into 2-aminoadenosine nucleoside in a protected (blocked) form or otherwise subsequently incorporated into an oligonucleotide and de-protected if necessary to form a final, desired compound. Substituent groups include groups covalently attached to the purine ring.

It will be appreciated by those skilled in the art that a 2-aminoadenine will typically be attached to a sugar moiety (e.g. 2-aminoadenosine). Such 2-amino modified adenosines can be incorporated into oligonucleotides in ways well known to persons of ordinary skill in the art, especially via automated methods. Other structures such as those employing modified sugars, modified nucleotide bond and otherwise are also useful herein.



(I)

Substituents at the amino functions of the adenine are represented as R_1,R_2, R_3 and R_4 in formula (I) which include hydrocarbyl groups such as alkyl or substituted alkyl, alkenyl or substituted alkenyl, alkynyl or substituted alkynyl, cycloalkyl, cycloalkenyl, cycloaralkyl, aryl, aralkyl or substituted aralkyl. Preferably, R_1 to R_4 are independently alkyl, alkenyl and alkynyl substituent , collectively hydrocarbyls, groups having from 1 to about 30 carbons, with 1 to about 10 carbons being particularly preferred. Preferably, the aryl groups have from 6 to about 14 carbons, and aralkyl groups have from 7 to about 30 carbons. The substituent groups listed above can themselves bear substituent groups such as alkoxy, hydroxyl, amine, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, or alkyl, aryl, alkenyl, or alkynyl groups, and ether groups. Examples of alkyl substitutions are further disclosed by Manoharan, M., *Antisense Research and Applications*, Crooke and Lebleu, eds., CRC Press, Boca Raton, 1993. Amine substituents include NH_2, polyalkylamines, aminoalkylamines, hydrazines i.e. (-NH-NH-), hydroxylamines i.e. (-NH-OH), semicarbazides i.e (-NH-C(O)-NH-NH_2), thiosemicarbazides i.e (-NH-C(S)-NH-NH_2), hydrazones i.e. (-N=NH), or hydrazides i.e. (-C(O)-$NHNH_2$), imidazoles, imidazole amides, alkylimidazoles, tetrazole, and triazole.

In a particulary preferred embodiment, R_1 is H and R_2 is a group selected from a substituted aminoalkyl, (especially $-(CH_2)_n-N[CH_2)_nNH_2]_{1-2}$, wherein n = 0 to 30, e.g. dimethylaminoethyl, $-(CH_2)_2-N[CH_2)_2NH_2]_{1-2}$ wherein n = 0 to 30, phthalimidoalkyl (e.g. phthalimidoethyl), imidazolylalkyl (e.g. imidazolylethyl) and piperazinylalkyl (e.g. piperazinylethyl).

R_1 and R_2, or R_3 and R_4 may optionally form a 5 to 13 membered ring or ring system optionally incorporating additional heteroatoms selected from N, O and S. Such rings or ring systems may include additional purine heterocycles. Preferred are rings without

additional heteroatoms and rings with a single N. Preferred are rings containing 5 to 7 members. Most preferred are rings with 6 members. The rings may also contain unsaturated bonds.

2-aminoadenosine phosphoroamidites and phospho-triesters are prepared according to Chollet, A., *et al.* (*Chemica Scripta* **1986**, *26*, 37-40) by protecting the N2 position of adenine with isobutyryl and the N6 position with 1-methyl-2,2-diethoxy pyrrolidine. They are also available through vendors such as TriLink Biotechnologies, Inc., San Diego, CA). The monomers are incorporated in an oligonucleotide chain using standard synthesis methods.

Methods for synthesizing oligonucleotides include conversion to the phosphoramidite followed by solution phase or solid phase chemistries. Representative solution phase techniques are described in United States Patent No. 5,210,264, issued May 11, 1993 and commonly assigned with this invention. Representative solid phase techniques are those typically employed for DNA and RNA synthesis utilizing standard phosphoramidite chemistry. (*see, e.g.*, Protocols For Oligonucleotides And Analogs, Agrawal, S., ed., Humana Press, Totowa, NJ, 1993.)

Equipment for such solid phase synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., *Helv. Chim. Acta,* **1995,** *78*, 486). It is also well known to use similar techniques and commercially available modified amidites and solid supports. Solid supports according to the invention include controlled pore glass (CPG)(available from Glen Research, Sterling, VA), oxalyl-controlled pore glass (*see, e.g.*, Alul, *et al.*, *Nucleic Acids Research* **1991**, *19*, 1527), TentaGel Support -- an aminopolyethyleneglycol derivatized support (*see, e.g.*, Wright, *et al.*, *Tetrahedron Letters* **1993**, *34*, 3373) or Poros -- a copolymer of polystyrene/ divinylbenzene. Many other solid supports are commercially available and amenable to the present invention.

An alternative method for synthesizing oligonucleotides incorporating 2-aminoadenosine is described herein and in US Ser. No. 09/144,883, filed September 1, 1998, commonly assigned and hereby incorporated by reference.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of a plurality of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

Specific examples of some preferred modified oligonucleotides envisioned for this invention include those containing phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioates (usually abbreviated in the art as P=S) and those with CH_2-NH-O-CH_2, CH_2-N(CH_3)-O-CH_2 [known as a methylene(methylimino) or MMI backbone], CH_2-O-N(CH_3)-CH_2, CH_2-N(CH_3)-N(CH_3)-CH_2 and O-N(CH_3)-CH_2-CH_2 backbones, wherein the native phosphodiester (usually abbreviated in the art as P=O) backbone is represented as O-P-O-CH_2. Also preferred are oligonucleotides having morpholino backbone structures such as those of Summerton and Weller, U.S. Patent 5,034,506.

Also preferred are oligonucleotides with NR-C(*)-CH_2-CH_2, CH_2-NR-C(*)-CH_2, CH_2-CH_2-NR-C(*), C(*)-NR-CH_2-CH_2 and CH_2-C(*)-NR-CH_2 backbones, wherein "*" represents O or S (known as amide and thioamide backbones; DeMesmaeker *et al.*, WO 92/20823, published November 26, 1992). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen *et al.*, *Science*, **1991**, *254*, 1497; U.S. Patent No. 5,539,082).

Other preferred modified oligonucleotides may contain one or more substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH_3, F, OCN, $OCH_2CH_2OCH_3$, $OCH_2CH_2O(CH_2)_nCH_3$, $O(CH_2)_nNH_2$ or $O(CH_2)_nCH_3$ where n is from 1 to about 10; O-R or O-R-O-R where R is C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl or aralkyl; O-substituted lower alkyl, Cl; Br; CN; CF_3; OCF_3; O-, S-, or N-alkyl; O-, S-, or N-

alkenyl; $SOCH_3$; SO_2CH_3; ONO_2; NO_2; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties.

Other substitutions at the 2' position include those disclosed in US Serial No. 09/016520, commonly assigned and herein incorporated by reference. Substitutions therein disclosed include aminooxy modifications, including O-aminooxyalkyl, O-alkylaminooxyalkyl and dialkylaminooxyethyl (e.g. dimethylaminooxyethyl) and are preferred. Another preferred modification includes 2'-O-methoxyethyl [which can be written as 2'-O-$CH_2CH_2OCH_3$, and is also known in the art as 2'-O-(2-methoxyethyl) or 2'-methoxyethoxy] (Martin, *et al.*, *Helv. Chim. Acta* **1995**, *78*, 486). Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-propoxy (2'-$OCH_2CH_2CH_3$) and 2'-fluoro (2'-F). A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of the 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

The oligonucleotides produced by this invention may additionally include other nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine and 5-methylcytosine, as well as synthetic nucleobases, e.g., 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N^6(6-aminohexyl)adenine and 2,6-diaminopurine (Kornberg, A., *DNA Replication*, 1974, W.H. Freeman & Co., San Francisco, **1974**, pp. 75-77; Gebeyehu, G., *et al., Nucleic Acids Res.* **1987**, *15*, 4513).

Another preferred additional or alternative modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more lipophilic moieties which enhance the cellular uptake of the oligonucleotide. Such lipophilic moieties may be linked to an oligonucleotide at several different positions on the oligonucleotide.

Some preferred positions include the 3' position of the sugar of the 3' terminal nucleotide, the 5' position of the sugar of the 5' terminal nucleotide, and the 2' position of the sugar of any nucleotide. The N^6 position of a purine heterocycle may also be utilized to link a lipophilic moiety to an oligonucleotide of the invention (Gebeyehu, G., *et al., Nucleic Acids Res.* **1987**, *15*, 4513). Such lipophilic moieties include but are not limited to a cholesteryl moiety (Letsinger *et al., Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6553), cholic acid (Manoharan *et al., Bioorg. Med. Chem. Let.* **1994**, *4*, 1053), a thioether, e.g., hexyl-*S*-tritylthiol (Manoharan *et al., Ann. N.Y. Acad. Sci.* **1992**, *660*, 306; Manoharan *et al., Bioorg. Med. Chem. Let.* **1993**, *3*, 2765), a thiocholesterol (Oberhauser *et al., Nucl. Acids Res.* **1992**, *20*, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al.* **1991**, *EMBO J.*, *10*, 111; Kabanov *et al., FEBS Lett.* **1990**, *259*, 327; Svinarchuk *et al., Biochimie* **1993**, *75*, 49), a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al., Tetrahedron Lett.* **1995**, *36*, 3651; Shea *et al., Nucl. Acids Res.* **1990**, *18*, 3777), a polyamine or a polyethylene glycol chain (Manoharan *et al., Nucleosides & Nucleotides* **1995**, *14*, 969), or adamantane acetic acid (Manoharan *et al., Tetrahedron Lett.* **1995**, *36*, 3651), a palmityl moiety (Mishra *et al., Biochim. Biophys. Acta* **1995**, *1264*, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.* **1996**, *277*, 923). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides, as disclosed in U.S. Patents No. 5,138,045, No. 5,218,105 and No. 5,459,255, the contents of which are hereby incorporated by reference.

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

enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Examples of chimeric oligonucleotides include but are not limited to "gapmers," in which three distinct regions are present, normally with a central region flanked by two regions which may be chemically equivalent to each other but are distinct from the gap. A preferred example of a gapmer is an oligonucleotide in which a central portion (the "gap") of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, while the flanking portions (the 5' and 3' "wings") are modified to have greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted).

Other chimeras include "wingmers," also known in the art as "hemimers," that is, oligonucleotides with two distinct regions. In a preferred example of a wingmer, the 5' portion of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, whereas the 3' portion is modified in such a fashion so as to have greater affinity for the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted), or vice-versa. In one embodiment, the oligonucleotides of the present invention contain a 2'-O-methoxyethyl (2'-O-$CH_2CH_2OCH_3$) modification on the sugar moiety of at least one nucleotide. This modification has been shown to increase both affinity of the oligonucleotide for its target and nuclease resistance of the oligonucleotide.

According to certain embodiments of the invention, one, a plurality, or all of the nucleotide subunits of the oligonucleotides of the invention may bear a 2'-O-methoxyethyl (-O-$CH_2CH_2OCH_3$) modification. Oligonucleotides comprising a plurality of nucleotide subunits having a 2'-O-methoxyethyl modification can have such a modification on any of the nucleotide subunits within the oligonucleotide, and may be chimeric oligonucleotides. Aside from or in addition to 2'-O-methoxyethyl modifications, oligonucleo-tides containing other modifications which enhance antisense efficacy, potency or target affinity are also preferred. Chimeric oligonucleotides comprising one or more such modifications are presently preferred. Oligonucleotides in accordance with this invention are from 5 to 50 nucleotides in length. In

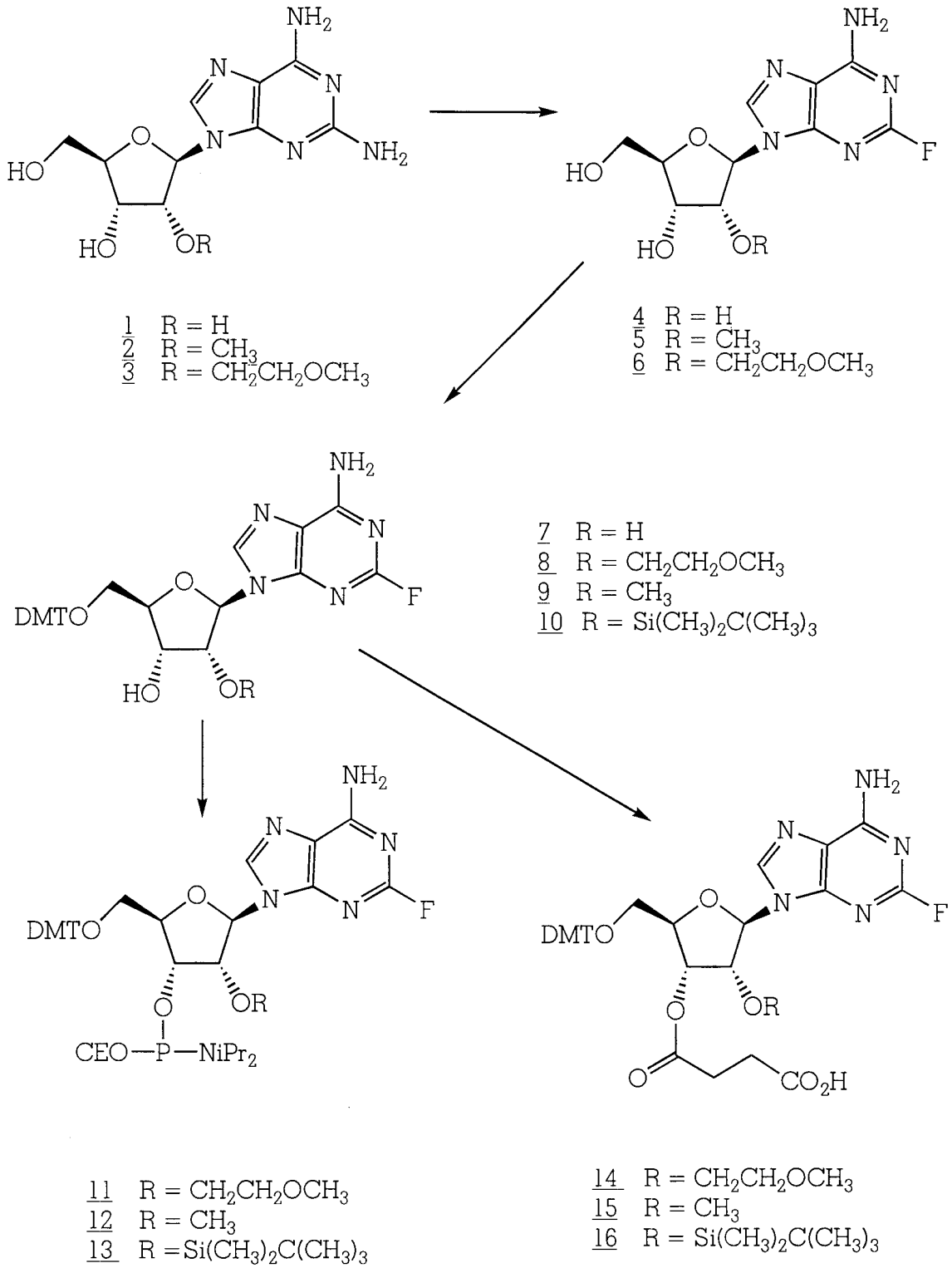
the context of this invention it is understood that this encompasses non-naturally occurring oligomers as hereinbefore described, having from 5 to 50 monomers.

Additional objects, advantages, and novel features of this invention will become apparent to those skilled in the art upon examination of the examples thereof provided below.

EXAMPLES

Examples 1-13 are depicted in Reaction scheme 1. Examples 14-20 are depicted in Reaction scheme 2. The underlined numbers in parentheses following the Example's title compound correspond to the compound numbers on the respective reaction schemes.

Reaction scheme 1



1 R = H
2 R = CH_3
3 R = $CH_2CH_2OCH_3$

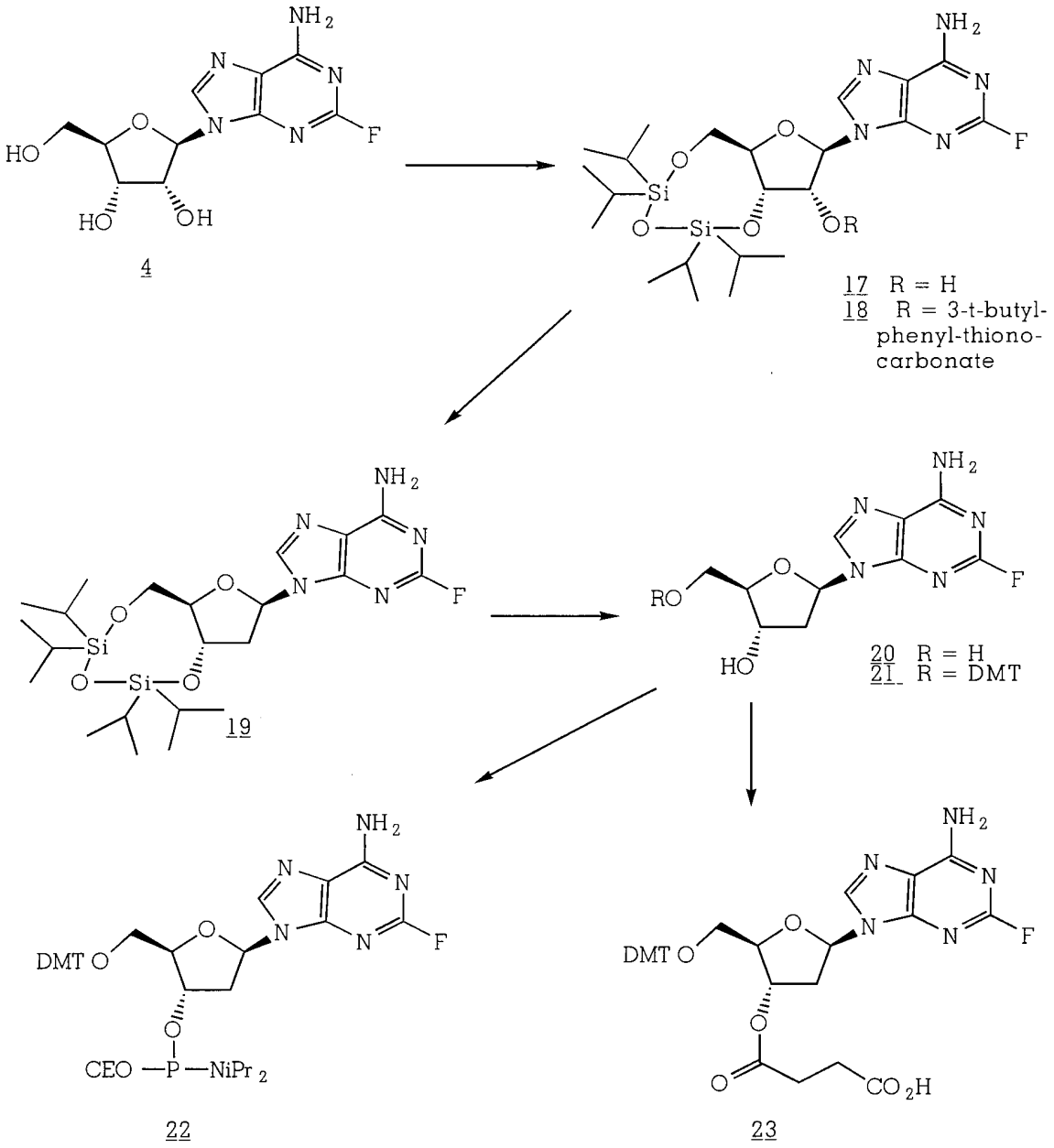
4 R = H
5 R = CH_3
6 R = $CH_2CH_2OCH_3$

7 R = H
8 R = $CH_2CH_2OCH_3$
9 R = CH_3
10 R = $Si(CH_3)_2C(CH_3)_3$

11 R = $CH_2CH_2OCH_3$
12 R = CH_3
13 R = $Si(CH_3)_2C(CH_3)_3$

14 R = $CH_2CH_2OCH_3$
15 R = CH_3
16 R = $Si(CH_3)_2C(CH_3)_3$

Reaction scheme 2



Example 1: Preparation of 2-fluoroadenosine (4)

A 2 L polyethylene bottle was equipped with a magnetic stirrer, thermometer, dry ice/acetone bath and a stream of argon gas. Anhydrous pyridine (750 mL) was added and the solution was cooled to -20°C. To this was added 70% hydrogen fluoride in pyridine (500 mL). 2,6-diaminopurine riboside (1, 2-aminoadenosine, 105 g, 0.372 mol, R.I. Chemical,

Orange, CA, Reliable Biopharmaceuticals, St. Louis, MO) was suspended in the liquid. Tert-butylnitrite (90 mL, 0.76 mol) was added in one portion and the reaction was stirred at 9-11°C until the reaction was complete as judged by TLC (3 h, Rf 0.20, starting material; Rf 0.40, product, ethyl acetate-methanol 4:1). Sodium bicarbonate (3 kg) was suspended with manual stirring in water (2 L) in a 20 L bucket. The reaction solution was slowly poured (to allow for evolution of carbon dioxide) into the aqueous layer with vigorous stirring. Ethyl acetate was added periodically in small portions to reduce foaming. The resulting suspension was extracted with ethyl acetate (5x 1 L). The organic layers were combined and concentrated to a solid. The solid was mostly dissolved in warm methanol (300 mL). The solution was cooled in a ice water bath and the resulting solid was collected, rinsed with methanol (2x100 mL) and dried under vacuum (1 mm Hg, 25°C, 24 h) to give 76 g of product as a gold solid, mp 214-215°C. The mother liquor was concentrated to a solid and recrystallized from water (600 mL) to give in three crops an additional 18.3 g of similar quality product for a total of 90.3 g (85%). H-NMR (DMSO-d6) d 3.4-3.8 (m, 2, 5'and 5"-H), 3.95 (m, 1, 4'-H), 4.14 (m, 1, 3'-H), 4.53 (m, 1, 2'-H), 5.04 (t, 1, 5'-OH), 5.23 (d, 1, 3'-OH), 5.50 (d, 1, 2'-OH), 5.80 (d, 1, 1'-H), 7.89 (br s, 2, NH_2), 8.37 (s, 1, 8-H).

Example 2: Preparation of 5'-O-(4,4'-dimethoxytriphenylmethyl)-2-fluoroadenosine (7)

2-Fluoroadenosine (4, 20.7 g, 0.0725 mol) and 4,4'-dimethoxytriphenylmethyl chloride (34.4g , 0.1016 mol) were dissolved in anhydrous pyridine (250 mL) at ambient temperature for 2 h. TLC (Rf 0.50, starting material; Rf 0.80, product, ethyl acetate-methanol 9:1) indicated only 80% conversion. An additional 7 g (0.021 mol) of the DMT chloride was added. TLC after 1 h indicated a complete reaction. The reaction was quenched by the addition of methanol (30 mL) and after 30 min, the reaction was concentrated under reduced pressure to an oil. The oil was partitioned between ethyl acetate (300 mL) and sat'd sodium bicarbonate solution (300 mL). The aqueous was extracted with more solvent (100 mL) and the combined organic layer was concentrated under reduced pressure to a small volume. A solid began to form. It was suspended

in a mixture of hexanes-ethyl acetate (300 mL, 1:1) and then collected by filtration, washed with the same mix (3x150 mL) and dried (1 mm Hg, 25°C, 24 h) to 26.2 g of product as light yellow crystal, mp sh 139 °C, melts 146-148 °C. The combined filtrate was concentrated and

purified by column chromatography (silica, 200 g) using a gradient of ethyl acetate in hexanes (70% to 80% to all ethyl acetate to 5% methanol in ethyl acetate). The appropriate fractions were combined, evaporated and dried as above to give 5.9 g more product as a foam for a total of 32.1 g (75.4%). H-NMR (DMSO-d6) d 3.22 (m, 2, 5'and 5"-H), 3.75 (s, 6, O-CH_3), 4.08 (m, 1, 4'-H), 4.30 (dd, 1, 3'-H), 4.63 (dd, 1, 2'-H), 5.26 (d, 1, 3'-OH), 5.60 (d, 1, 2'-OH), 5.87 (d, 1, 1'-H), 6.87 (m, 4, arom.), 7.1-7.4 (m, 9, arom.), 7.92 (br s, 2, NH_2), 8.26 (s, 1, 8-H).

Example 3: Preparation of 2'-O-tert-butyl-dimethylsilyl-5'-O-(4,4'-dimethoxytriphenylmethyl)-2-fluoroadenosine (10)

5'-O-(4,4'-dimethoxytriphenylmethyl)-2-fluoroadenosine (7, 14 g, 0.046 mol), tert-butyldimethylsilyl chloride (9.1 g, 0.060 mol), anhydrous pyridine (7.8 mL), silver nitrate (9.0 g, 0.053 mol) were dissolved in anhydrous tetrahydro-furan (125 mL). The reaction was stirred at ambient temperature under an argon atmosphere for 3 h. TLC indicated a complete reaction (Rf 0.10, starting material; Rf 0.50, product, Rf 0.45, 3' isomer, hexanes-acetone 6:4). The reaction was quenched by the addition of water (25 mL) and then concentrated to an oil under reduced pressure. The residue was dissolved in a minimum of dichloromethane and applied onto a silica gel column (450 g) and eluted with hexanes-acetone (7:3). The appropriate fractions were combined, concentrated and then redissolved in a minimum of warm ethyl acetate to give a precipitate upon cooling. The solid was collected by filtration, washed with ethyl acetate (2x20 mL) and dried (1 mm Hg, 25 °C, 24 h) to 8.5 g (50%) of product as white microcrystalline powder, mp 103-107 °C. H-NMR (DMSO-d6) d 0.0 (s, 6, Si-CH_3), 0.78 (s, 9, Si-t-but), 3.28 (m, 2, 5'and 5"-H), 3.74 (s, 6, O-CH_3), 4.09 (m, 1, 4'-H), 4.21 (dd, 1, 3'-H), 4.73 (dd, 1, 2'-H), 5.19 (d, 1, 3'-OH), 5.86 (d, 1, 1'-H), 6.85 (d, 4, arom.), 7.2-7.4 (m, 9, arom.), 7.90 (br s, 2, NH_2), 8.25 (s, 1, 8-H). F-NMR (DMSO-d6) d -53.0 (s).

Example 4: Preparation of [5'-O-(4,4'-Dimethoxytrityl)-2'-O-tert-butyldimethylsilyl-2-fluoroadenosin-3'-O-yl]-N,N-diisopropylaminocyanoethoxy phosphoramidite (13)

5'-O-(4,4'-Dimethoxytrityl)-2'-O-tert-butyldimethylsilyl-2-fluoroadenosine (10, 5.0 g, 0.0071 mol), 2-cyanoethyl diisopropylchlorophosphoroamidite (5.6 mL, 0.025 mol), N-methyl imidazole (0.3 mL), collidine (7.2 mL) were dissolved in anhydrous tetrahydrofuran (75 mL) with stirring at ambient temperature under an argon atmosphere. After 2.5 h, TLC

indicated a complete reaction (Rf 0.30, starting material; Rf 0.50 product diastereomers, hexanes-acetone 6:4). The reaction was diluted with ethyl acetate (200 mL) and washed with sat'd sodium bicarbonate solution (300 mL). The aqueous layer was back-extracted with ethyl acetate (75 mL). The combined organic layer was washed with brine (200 mL), concentrated under reduced pressure to a thin oil and then directly applied to a silica gel column (100 g). The product was eluted with a mixture of hexanes-acetone-triethylamine (70:29:1). The appropriate fractions were combined, concentrated under reduced pressure, coevaporated with anhydrous acetonitrile and dried (1 mm Hg, 25 °C, 24 h) to 3.3 g (52%) of white foam. H-NMR ($CDCl_3$) d 0.05 (d, 6, Si-CH_3), 0.82 (s, 9, Si-t-but), 1.0-1.4 (m, 14, N-iPr), 2.33 and 2.63 (t and t, 2, CH_2CN, diastereomers), 3.3-4.0 (m, 4, 5'and 5"-H and CH_2CH_2CN), 3.78 (s, 6, O-CH_3), 4.3-4.5 (m, 2, 3' and 4'-H), 4.90 (m, 1, 2'-H), 5.92 (dd, 1, 1'-H), 6.50 (br s, 2, NH_2), 6.82 (m, 4, arom.), 7.2-7.5 (m, 9, arom.), 8.15 (d, 1, 8-H). F-NMR ($CDCl_3$) d -51.6 (s). P-NMR ($CDCl_3$) d 150.0 and 151.4 (s and s, diastereomers).

Example 5: Preparation of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-succinyl-2'-O-tert-butyldimethylsilyl-2-fluoroadenosine (16)

5'-O-(4,4'-Dimethoxytrityl)-2'-O-tert-butyldimethylsilyl-2-fluoroadenosine (**10**, 1.0 g, 1.42 mmol), dimethylaminopyridine (0.32 g, 2.8 mmol) and succinic anhydride (0.87 g, 8.7 mmol) were dissolved in anhydrous pyridine (35 mL) and stirred at ambient temperature under an argon atmosphere until the reaction was complete (6 h) by TLC (Rf 0.50, starting material; Rf 0.17, product, ethyl acetate-hexanes 1:1). The reaction was quenched by the addition of water (10 mL) and then concentrated under reduced pressure to an oil. The oil was partitioned between ethyl acetate (200 mL) and sat'd sodium bicarbonate solution (200 mL). The aqueous layer was back extracted with more ethyl acetate. The combined organic phase was concentrated under reduced pressure and applied to a silica gel column (20 g). The product was eluted with a mix of ethyl acetate-hexanes (1:1). The appropriate fractions were combined, concentrated to a foam, coevaporated with acetonitrile (10 ml) and dried (1 mm Hg, 25°C, 24 h) to give product 0.70 g (62%) as a white foam. H-NMR (DMSO-d6) d -0.28 and 0.09 (s and s, 6, Si-CH_3, rotamers), 0.68 (s, 9, Si-t-but), 2.4-2.6 (m, 6, CH_2CH_2 CO_2H and 5'and 5"-H), 3.74 (s, 6, O-CH_3), 4.22 (m, 1, 4'-H), 5.14 (dd, 1, 2'-H),

5.31 (dd, 1, 3'-H), 5.85 (d, 1, 1'-H), 6.86 (d, 4, arom.), 7.2-7.45 (m, 9, arom.), 7.95 (br s, 2, NH_2), 8.31 (s, 1, 8-H), 12.24 (br s, 1, CO_2H).

Example 6: Preparation of 2'-O-(2-methoxyethyl)-2-fluoroadenosine (6)

A 2 L polyethylene bottle was equipped with a magnetic stirrer, thermometer, dry ice/acetone bath and a stream of argon gas. Anhydrous pyridine (350 mL) was added and the solution was cooled to -20°C. To this was added 70% hydrogen fluoride in pyridine (200 mL) while maintaining the temperature below 0 °C. 2'-O-(2-methoxyethyl)-2-aminoadenosine, (**3**, 42 g, 0.124 mol) was prepared as described in US Patent 5,506,351, herein incorporated by reference, using methyloxyethyl bromide (2-bromoethyl methyl ether) in place of either iodomethane or iodopropane. The product was dissolved in the solution. *Tert*-butylnitrite (56 mL, 0.47 mol) was added in one portion and the reaction was stirred at 5-16 °C until the reaction was complete as judged by TLC (2 h, Rf 0.40, starting material; Rf 0.60, product, ethyl acetate-methanol 6:4). Sodium bicarbonate (1400 g) was suspended with manual stirring in water (1.5 L) in a 20 L bucket. The reaction solution was slowly poured (to allow for evolution of carbon dioxide) into the aqueous layer with vigorous stirring. The resulting solution (pH 7-8) was extracted with ethyl acetate (3x800 mL). The organic layers were combined and concentrated to an oil which was triturated with methanol (50 mL) to form a solid. The solid was collected by filtration and washed with ethyl acetate (3x50 mL) to 27.9 g of light tan crystals. The filtrate was concentrated under reduced pressure and redissolved in a minimum of methanol and allowed to stand at ambient temperature overnight. The second crop was collected as above to give 5.1 g. The mother liquor was concentrated again, then redissolved in a minimum of ethyl acetate and applied unto a silica gel column (100 g). The product was eluted with ethyl acetate. The appropriate fractions were combined and concentrated to 4.8 g of foam for a total of 37.8 g (89%) of product. A sample was recrystallized from methanol for elemental analysis to give white crystals, mp 152-154 °C. Cd: C, 45.80, H, 5.28; N, 20.40. Fd: C, 45.48; H, 5.12; N, 20.20. H-NMR (DMSO-d6) d 3.16 (s, 3, CH_3OCH_2) 3.35-3.75 (m, 6, 5'and 5"-H and OCH_2CH_2O), 3.97 (dd, 1, 4'-H), 4.31 (dd, 1, 3'-H), 4.48 (dd, 1, 2'-H), 5.12 (t, 1, 5'-OH), 5.18 (d, 1, 3'-OH), 5.91 (d, 1, 1'-H), 7.92 (br s, 2, NH_2), 8.39 (s, 1, 8-H).

Example 7: Preparation of 2'-O-(2-methoxyethyl)-5'-O-(4,4'-dimethoxytriphenylmethyl)-2-fluoroadenosine (8)

2'-O-(2-Methoxyethyl)-2-fluoroadenosine (6, 37.0 g, 0.108 mol) and 4,4'-dimethoxytriphenylmethyl chloride (40.2 g , 0.118 mol) were dissolved in anhydrous pyridine (250 mL) at ambient temperature for 1 h. TLC (Rf 0.50, starting material; Rf 0.80, product, ethyl acetate-methanol 4:1) indicated only 70% conversion. An additional 5 g (0.015 mol) of the DMT chloride was added. TLC after 1 h indicated a complete reaction. The reaction was quenched by the addition of methanol (30 mL) and after 30 min, the reaction was concentrated under reduced pressure to an oil. The oil was partitioned between ethyl acetate (300 mL) and sat'd sodium bicarbonate solution (300 mL). The aqueous was extracted with more solvent (300 mL) and the combined organic layer was concentrated under reduced pressure to a small volume and applied onto a silica gel column (700 g). The product was eluted with ethyl acetate-hexanes (9:1) and then ethyl acetate to obtain all the product. The appropriate pure fractions were combined, concentrated causing a precipitate to form. The product was dried (1 mm Hg, 25°C, 24 h) to 49.4 g yellow crystalline solid, mp 88-96 °C. Impure fractions were combined and recolumned as above to give an additional 4.8 g for a total of 54.2 g (82%) as a light yellow foam. H-NMR (DMSO-d6) d 3.18 (s, 3, CH_3OCH_2), 3.20-3.80 (m, 6, 5'and 5"-H and OCH_2CH_2O), 3.75 (s, 6, O-CH_3), 4.05 (m, 1, 4'-H), 4.40 (dd, 1, 3'-H), 4.59 (dd, 1, 2'-H), 5.22 (d, 1, 3'-OH), 5.95 (d, 1, 1'-H), 6.83 (m, 4, arom.), 7.15-7.4 (m, 9, arom.), 7.93 (br s, 2, NH_2), 8.27 (s, 1, 8-H). F-NMR (DMSO-d6) d -52.8 (s).

Example 8: Preparation of [5'-O-(4,4'-Dimethoxytrityl)-2'-O-(2-methoxyethyl)-2-fluoroadenosin-3'-O-yl]-N,N-diisopropylaminocyanoethoxy phosphoramidite (11)

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(2-methoxyethyl)-2-fluoroadenosine (8, 10 g, 0.16 mol), 2-cyanoethyl tetraisopropylphosphorodiamidite (6.9 g, 0.22 mol), diisopropylamine tetrazolide (0.84 g, 0.0049 mol) were dissolved in anhydrous dichloromethane (200 mL) and allowed to stir at ambient temperature under an argon atmosphere for 16 h. TLC indicated a complete reaction (Rf 0.20, starting material; Rf 0.55, product diastereomers, ethyl acetate-methanol 9:1). The reaction was washed with sat'd sodium bicarbonate solution. The aqueous layer was back-extracted with dichloromethane (100 mL). The combined organic layer was dried over sodium sulfate, concentrated to a thin

oil and then directly applied to a silica gel column (200 g). The product was eluted with ethyl acetate-hexanes-triethylamine (80:19:1). The appropriate fractions were combined, concentrated under reduced pressure, coevaporated with anhydrous acetonitrile and dried (1 mm Hg, 25 °C, 24 h) to 9.3 g (70%) of product as a light yellow foam. H-NMR ($CDCl_3$) d 1.0-1.3 (m, 14, N-iPr), 2.39 and 2.65 (t and t, 2, CH_2CN, diastereomers), 3.23 (s, 3, CH_3O-CH_2), 3.3-4.0 (m, 8, 5'and 5"-H and CH_2CH_2CN and OCH_2CH_2O), 3.78 (s, 6, O-CH_3), 4.35 (m, 1, 4'-H), 4.60 (m, 1, 3'-H), 4.78 (m, 1, 2'-H), 5.90 (br s, 2, NH_2), 6.02 (dd, 1, 1'-H), 6.80 (m, 4, arom.), 7.2-7.5 (m, 9, arom.), 8.00 (d, 1, 8-H). P-NMR ($CDCl_3$) d 150.7and 151.0 (s and s, diastereomers).

Example 9: Preparation of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-succinyl-2'-O-(2-methoxyethyl)-2-fluoroadenosine (14)

5'-O-(4,4'-Dimethoxytrityl)-2'-O-(2-methoxyethyl)-2-fluoroadenosine (**8**, 1.0 g, 1.63 mmol), dimethylaminopyridine (0.080 g, 0.66 mmol) and succinic anhydride (0.65 g, 6.5 mmol) were dissolved in anhydrous pyridine (10 mL) and stirred at ambient temperature under an argon atmosphere until the reaction was complete (6 h) by TLC (Rf 0.60, starting material; Rf 0.40, product, ethyl acetate-methanol 6:4). The reaction was quenched by the addition of water (10 mL) and then concentrated under reduced pressure to an oil. The oil was partitioned between ethyl acetate (75 mL) and 20% aqueous citric acid (75 mL). The organic phase was washed with water (2x75 mL), dried over sodium sulfate, concentrated under reduced pressure, coevaporated with acetonitrile (100 mL) and dried (1 mm Hg, 25°C, 24 h) to 1.0 g (83%) of product as a white foam. H-NMR (DMSO-d6) d 2.4-2.6 (m, 4, $CH_2CH_2CO_2H$), 3.55 (m, 2, 5'and 5"-H), 3.75 (s, 6, O-CH_3), 4.22 (m, 1, 4'-H), 4.98 (dd, 1, 2'-H), 5.42 (dd, 1, 3'-H), 5.95 (d, 1, 1'-H), 6.84 (m, 4, arom.), 7.2-7.45 (m, 9, arom.), 7.97 (br s, 2, NH_2), 8.32 (s, 1, 8-H), 12.23 (br s, 1, CO_2H).

Example 10: Preparation of 2'-O-methyl-2-fluoroadenosine (5)

A 2 L polyethylene bottle was equipped with a magnetic stirrer, thermometer, dry ice/acetone bath and a stream of argon gas. Anhydrous pyridine (200 mL) was added and the solution was cooled to -20°C. To this was added 70% hydrogen fluoride in pyridine (100 mL) while maintaining the temperature below 0 °C. 2'-O-Methyl-2-aminoadenosine, (**2**, 30

g, 0.101 mol, R.I. Chemical, Orange, CA, Reliable Bio-pharmaceuticals, St. Louis, MO) was dissolved in the solution. Tert-butylnitrite (42 mL, 0.35 mol) was added in one portion and the reaction was stirred at 5-13 °C until the reaction was complete as judged by TLC (2 h, Rf 0.15, starting material; Rf 0.28, product, ethyl acetate-methanol 9:1). Sodium bicarbonate (600 g) was suspended with manual stirring in water (1 L) in a 20 L bucket. The reaction solution was slowly poured (to allow for evolution of carbon dioxide) into the aqueous layer with vigorous stirring. The resulting solution (pH 7-8) was extracted with ethyl acetate (5x400 mL). The organic layers were combined and concentrated to an oil which was triturated with methanol (70 mL) to form a solid. The solid was collected by filtration and washed with ethyl acetate (3x50 mL) and dried (1 mm Hg, 25°C, 24 h) to 16.5 g of product as a cream colored microcrystalline solid, mp 239-241 °C. The filtrate was concentrated under reduced pressure to about half volume and a second crop was collected as above to give as additional 1.0 g or 17.5 g total (58%). More product remained in the mother liquor. H-NMR (DMSO-d6) d 3.36 (s, 3, 2'-O-CH_3) 3.50-3.75 (m, 2, 5'and 5"-H), 3.98 (m, 1, 4'-H), 4.32 (m, 2, 3'-H and 2'-H), 5.14 (t, 1, 5'-OH), 5.31 (d, 1, 3'-OH), 5.92 (d, 1, 1'-H), 7.92 (br s, 2, NH_2), 8.40 (s, 1, 8-H).

Example 11: Preparation of 2'-O-Methyl-5'-O-(4,4'-dimethoxy triphenylmethyl)-2-fluoroadenosine (9)

2'-O-Methyl-2-fluoroadenosine (5, 16.5 g, 0.055 mol) and 4,4'-dimethoxytriphenylmethyl chloride (24.3 g , 0.0717 mol) were dissolved in anhydrous pyridine (150 mL) at ambient temperature for 1 h. TLC (Rf 0.30, starting material; Rf 0.60, product, ethyl acetate-methanol 9:1) indicated only 70% conversion. An additional 10 g (0.030 mol) of the DMT chloride was added. TLC after 1 h indicated a complete reaction. The reaction was quenched by the addition of methanol (50 mL) and after 30 min, the reaction was concentrated under reduced pressure to an oil. The oil was taken up in ethyl acetate (50 mL) and adsorbed onto silica gel (60 g). The silica was dried under reduced pressure to a free flowing powder and placed on top of a silica gel column (200 g). The product was eluted with a gradient of ethyl acetate in hexanes (50% to 100 % ethyl acetate). The appropriate fractions were combined, concentrated and dried (1 mm Hg, 25 °C, 24 h) to 19.1 g (58%) of product as a yellow foam. H-NMR (DMSO-d6) d 3.22 (m, 2, 5'-H and 5"-H), 3.38 (s, 3, 2'-O-CH_3),

3.75 (s, 6, O-CH_3), 4.05 (m, 1, 4'-H), 4.41 (m, 2, 2'-H and 3'-H), 5.31 (d, 1, 3'-OH), 5.97 (d, 1, 1'-H), 6.83 (m, 4, arom.), 7.15-7.4 (m, 9, arom.), 7.93 (br s, 2, NH_2), 8.25 (s, 1, 8-H). F-NMR (DMSO-d6) d -52.8 (s).

Example 12: Preparation of [5'-O-(4,4'-Dimethoxytrityl)-2'-O-methyl-2-fluoroadenosin-3'-O-yl]-N,N-diisopropylaminocyanoethoxy phosphoramidite (12)

5'-O-(4,4'-Dimethoxytrityl)-2'-O-methyl-2-fluoroadenosine (9, 10 g, 0.17 mol), 2-cyanoethyl tetra-isopropylphosphorodiamidite (7.0 g, 0.23 mol), diisopropyl-amine tetrazolide (0.85 g, 0.0050 mol) were dissolved in anhydrous dichloromethane (100 mL) and allowed to stir at ambient temperature under an argon atmosphere for 16 h. TLC indicated a complete reaction (Rf 0.40, starting material; Rf 0.65 and 0.70, product diastereomers, ethyl acetate). With no workup, the reaction was added directly to a silica gel column (200 g) and eluted with a mixture of ethyl acetate-hexanes-triethylamine (60:39:1). The product containing fractions were combined and concentrated to 10.2 g of foam. P-NMR indicated an 8% impurity peak at 7 ppm. The product was recolumned using ethyl acetate-triethylamine (99:1) to give 8 g of pure product and 2 g of 30% impure product which was discarded. The pure product was coevaporated with acetonitrile (50 mL) and dried (1 mm Hg, 25 °C, 24 h) to 7.5 g (56%) of product as white foam. H-NMR ($CDCl_3$) d 1.0-1.3 (m, 14, N-iPr), 2.37 and 2.66 (t and t, 2, CH_2CN, diastereomers), 3.2-4.0 (m, 4, 5'and 5"-H and CH_2CH_2CN), 3.49 (s, 3, 2'-O-CH_3), 3.78 (s, 6, O-CH_3), 4.3-4.7 (m, 3, 2'-H, 3'-H and 4'-H), 6.02 (dd, 1, 1'-H), 6.51 (br s, 2, NH_2), 6.82 (m, 4, arom.), 7.2-7.5 (m, 9, arom.), 7.98 (d, 1, 8-H). P-NMR ($CDCl_3$) d 150.9 and 151.5 (s and s, diastereomers). F-NMR ($CDCl_3$) d -51.7 (s).

Example 13: Preparation of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-succinyl-2'-O-methyl-2-fluoroadenosine (15)

5'-O-(4,4'-Dimethoxytrityl)-2'-O-methyl-2-fluoro-adenosine (9, 2.0 g, 3.32 mmol), dimethylaminopyridine (0.81 g, 6.6 mmol) and succinic anhydride (1.3 g, 13.3 mmol) were dissolved in anhydrous pyridine (10 mL) and stirred at ambient temperature under an argon atmosphere until the reaction was complete (2 h) by TLC (Rf 0.90, starting material; Rf 0.30, product, ethyl acetate-methanol 6:4). The reaction was quenched by the addition of water (30 mL) and then concentrated under reduced pressure to an oil. The oil was partitioned

between ethyl acetate (100 mL) and 20% aqueous citric acid (100 mL). The organic phase was washed with water (2x100 mL), dried over sodium sulfate, concentrated under reduced pressure. TLC indicated impurities so the product was dissolved in a minimum of ethyl acetate, applied on a silica gel column (30 g) and washed with ethyl acetate-methanol (95:5) and then eluted with ethyl acetate-methanol (6:4). The product containing fractions were combined, concentrated under reduced pressure, coevaporated with acetonitrile (20 mL) and triethylamine (5 mL) and dried (1 mm Hg, 25°C, 24 h) to 2.1 g (90%) of the triethylamine salt of the product as a white foam. H-NMR (DMSO-d6) d 0.96 (t, 9, CH_3CH_2N), 2.4-255 (m, 10, $CH_2CH_2CO_2H$ and NCH_2CH_3), 3.26 (s, 3, 2'-O-CH_3), 3.40 (m, 2, 5'and 5"-H), 3.74 (s, 6, O-CH_3), 4.22 (m, 1, 4'-H), 4.82 (dd, 1, 2'-H), 5.42 (dd, 1, 3'-H), 5.96 (d, 1, 1'-H), 6.83 (m, 4, arom.), 7.2-7.45 (m, 9, arom.), 7.95 (br s, 2, NH_2).

Example 14: Preparation of 3',5'-O-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl-2-fluoroadenosine (17)

2-Fluoroadenosine (4, 22.5 g, 0.0789 mol) and 1,3-dichloro-(1,1,3,3-tetraisopropyl-1,3-disiloxane (28.8 g, 0.913 mol) were dissolved in anhydrous pyridine (150 mL) and stirred under an argon atmosphere at ambient temperature until the reaction was complete (3 h) by TLC (Rf 0.05,starting material; Rf 0.25, product; hexanes-ethyl acetate 1:1). The reaction was quenched with methanol (50 mL). After 20 min, the reaction was concentrated under reduced pressure to an oil which in turn was partitioned between ethyl acetate (400 mL) and a sat'd sodium bicarbonate solution (500 mL). The aqueous layer was extracted with more ethyl acetate (200 mL). The organic layers were combined, concentrated under reduced pressure and the resulting oil was applied onto a silica gel column (1.5 kg). The column was eluted with ethyl acetate until the product started to appear and then the polarity was increased by the addition of 5% methanol to drive off the balance of the product. The appropriate fraction were combined, concentrated under reduced pressure and dried (1 mm Hg, 25 °C, 24 h) to a foam, 62 g which was still heavily contaminated with silyl reagent (theory 39.6 g). NMR was consistent with structure and this material was used as is in the next step.

Example 15: Preparation of 3',5'-O-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)-2'-O-(3-tert-butylphenylthionocarbonyl)-2-fluoroadenosine (18)

3',5'-O-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl-2-fluoroadenosine (17, crude 62 g, theory 39.6 g, 0.79 mol), 3-tert-butylphenyl chlorothionoformate (22.6 g, 0.10 mol), dimethylaminopyridine (15.4 g, 0.126 mol) were dissolved in anhydrous dichloromethane (500 mL). The reaction was allowed to stir under an argon atmosphere at ambient temperature until complete (2 h) by TLC (Rf 0.25, starting material; Rf 0.50, product; hexanes-ethyl acetate 1:1). The reaction was quenched by the addition of a sat'd sodium bicarbonate solution (450 mL). The layers were separated and the aqueous layer was extracted with more dichloro-methane (200 mL). The combined organic layers were concentrated under reduced pressure to an oil which in turn was redissolved in a minimum of ethyl acetate. A crystalline precipitate formed. This was collected, washed with ethyl acetate (2x100 mL) and dried (1 mm Hg, 25 °C, 24 h) to a white solid, 25.2 g, mp 226-228 °C. The mother liquor was concentrated and the resulting oil was purified by silica gel chromatography (hexanes-ethyl acetate, 7:3) to give another 10.6 g of product as a foam. The combined product weighed 35.8 g (64% two-step yield). H-NMR (DMSO-d6) d 1.05 (m, 28, Si-iPr), 1.30 (s, 9, t-butyl), 3.9-4.1 (m, 4, 3'-H, 4'-H, 5'and 5"-H), 5.37 (dd, 1, 2'-H), 6.36 (s, 1, 1'-H), 6.43 (d, 1, 3'-OH), 6.95-7.5 (m, 4, arom.), 7.98 (br s, 2, NH_2), 8.28 (s, 1, 8-H).

Example 16: Preparation of 3',5'-O-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)-2'-deoxy-2-fluoroadenosine (19)

3',5'-O-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)-2'-O-(3-tert-butylphenylthionocarbonyl)-2-fluoroadenosine (18, 35.7 g, 0.50 mol) was dissolved in toluene (600 mL). Tributyltin hydride (28 g, 0.96 mol) and AIBN (2,2'-azobisisobutyronitrile, 7.6 g, 0.046 mol) were added and the solution was heated to reflux with stirring for 30 min. TLC indicated a complete reaction (Rf 0.50, starting material; Rf 0.35, product; hexanes-ethyl acetate 1:1). The reaction was allowed to cool and then it was diluted with methanol (100 mL). The solution was concentrated under reduced pressure to an oil which in turn was applied onto a silica gel column (700 g) and eluted with hexanes and then a mixture of hexanes-ethyl acetate (7:3). The product containing fractions were combined, concentrated under reduced pressure to a solid paste which in turn was triturated with hexanes (300 mL),

collected, dried (1 mm Hg, 25 °C, 24 h) to 20.8 g of product as a white small crystals, mp 201-202 °C. The filtrate was concentrated and recolumned to give an additional 1.5 g of similar material for a total of 22.3 g (86.5%). H-NMR (DMSO-d6) d 1.05 (m, 28, Si-iPr), 2.5-2.9 (m, 2, 2'-CH_2), 3.6-3.9 (m, 4, 3'-H, 4'-H, 5'and 5"-H), 5.12 (dd, 1, 3'-H), 6.22 (dd, 1, 1'-H), 7.83 (br s, 2, NH_2), 8.22 (s, 1, 8-H).

Example 17: Preparation of 2'-deoxy-2-fluoroadenosine (20)

3',5'-O-(1,1,3,3-Tetraisopropyl-1,3-disiloxanediyl)-2'-deoxy-2-fluoroadenosine (**19**, 22.2 g, 0.46 mol) was dissolved in anhydrous tetrahydrofuran (150 mL). Triethylamine (10 mL, 0.72 mol) and triethylamine trihydrofluoride (20 mL, 0.122 mol) were added and the reaction was stirred at ambient temperature until the reaction was complete (3 h) by TLC (Rf 0.90, starting material; Rf 0.20, product; ethyl acetate-methanol 9:1). The reaction was concentrated under reduced pressure to an oily solid. The residue was redissolved in a minimum of methanol and stirred with Dowex-50 beads (sulfonic acid resin, H+ form, 150 g of dry weight). The solution was filtered, concentrated to an oil and applied to a silica gel column (400 g). The product was eluted with a gradient of methanol in ethyl acetate (0-20%). The appropriate fractions were combined, concentrated under reduced pressure and dried (1 mm Hg, 25 °C, 24 h) to a solid, 12.1 g (98%), mp darkens above 178 °C but melts above 250 °C. H-NMR (DMSO-d6) d 2.2-2.3 and 2.6-2.75 (m, 2, 2'-CH_2),3.4-3.7 (m, 2, 5'and 5"-H), 3.85 (m, 1, 4'-H), 4.40 (m, 1, 3'-H), 4.98 (t, 1, 5'-OH), 5.35 (d, 1, 3'-OH), 6.23 (t, 1, 1'-H), 7.83 (br s, 2, NH_2), 8.34 (s, 1, 8-H).

Example 18: Preparation of 5'-O-(4,4'-Dimethoxytrityl)-2'-deoxy-2-fluoroadenosine (21)

2'-deoxy-2-fluoroadenosine (**20**, 12 g, 0.45 mol) and dimethoxytrityl chloride (18.1 g, 0.53 mol) were dissolved in anhydrous pyridine (100 mL) and stirred at ambient temperature until the trace amount of the starting material remaining matched the bis DMT impurity forming (3 h) as monitored by TLC (Rf 0.20, starting material; Rf 0.60, product; Rf 0.85, bis DMT product, ethyl acetate-methanol 9:1). The reaction was quenched by the addition of methanol (50 mL) and then concentrated under reduced pressure to an oil. The residue was partitioned between ethyl acetate (250 mL) and sat'd sodium bicarbonate (250

mL). The aqueous layer was extracted with ethyl acetate (100 mL) once more and the combined extracts were concentrated under reduced pressure. The resulting oil was purified by silica gel chromatography (400 g) using a gradient of methanol in ethyl acetate (0-5%). The appropriate fractions were combined, concentrated under reduced pressure, coevaporated with anhydrous acetonitrile (200 mL) and dried (1 mm Hg, 25 °C, 24 h) to a foam, 23.2 g (90%). The product can be crystallized from dichloromethane to give white crystals, mp 239-243 °C. H-NMR (DMSO-d6) d 2.2-2.4 and 2.7-2.9 (m, 2, 2'-CH_2), 3.18 (m, 2, 5'-H and 5"-H), 3.75 (s, 6, O-CH_3), 3.98 (m, 1, 4'-H), 4.43 (m, 1, 3'-H), 5.38 (d, 1, 3'-OH), 6.15 (t, 1, 1'-H), 6.83 (m, 4, arom.), 7.15-7.4 (m, 9, arom.), 7.83 (br s, 2, NH_2), 8.23 (s, 1, 8-H). F-NMR (DMSO-d6) d -53.5 (s).

Example 19: Preparation of [5'-O-(4,4'-Dimethoxytrityl)-2'-deoxy-2-fluoroadenosin-3'-O-yl]-N,N-diisopropylamino cyanoethoxy phosphoramidite (22)

5'-O-(4,4'-Dimethoxytrityl)-2'-deoxy-2-fluoroadenosine (**21**, 10 g, 0.18 mol), 2-cyanoethyl tetraisopropyl phosphorodiamidite (7.6 g, 0.25 mol), diisopropylamine tetrazolide (0.93 g, 0.0054 mol) were dissolved in anhydrous dichloromethane (100 mL) and allowed to stir at ambient temperature under an argon atmosphere for 16 h. TLC indicated a complete reaction (Rf 0.30, starting material; Rf 0.60 and 0.65, product diastereomers, ethyl acetate). The reaction was concentrated under reduced pressure to a thin oil and then directly applied to a silica gel column (200 g). The product was eluted with ethyl acetate-triethylamine (99:1). The appropriate fractions were combined, concentrated under reduced pressure, coevaporated with anhydrous acetonitrile and dried (1 mm Hg, 25°C, 24 h) to 9.8 g (73%) of white foam. H-NMR ($CDCl_3$) d 1.0-1.3 (m, 14, N-iPr), 2.45 and 2.63 (t and t, 2, CH_2CN, diastereomers), 2.80 (m, 2, 2'-CH_2), 3.3-4.0 (m, 4, 5'and 5"-H and CH_2CH_2CN), 3.78 (s, 6, O-CH_3), 4.25 (m, 1, 4'-H), 4.77 (m, 1, 3'-H), 6.36 (dd, 1, 1'-H), 6.50 (br s, 2, NH_2), 6.82 (m, 4, arom.), 7.2-7.5 (m, 9, arom.), 7.98 (d, 1, 8-H). P-NMR ($CDCl_3$) d 149.5 (s, diastereomers don't resolve).

Example 20: Preparation of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-succinyl-2'-deoxy-2-fluoroadenosine (23)

5'-O-(4,4'-Dimethoxytrityl)-2'-deoxy-2-fluoroadenosine (**21**, 2.3 g, 4.03 mmol), dimethylaminopyridine (0.16 g, 1.3 mmol) and succinic anhydride (1.7 g, 17 mmol) were dissolved in anhydrous pyridine (20 mL) and stirred at ambient temperature under an argon atmosphere until the reaction was complete (6 h) by TLC (Rf 0.80, starting material; Rf 0.20, product, ethyl acetate-methanol 4:1). The reaction was quenched by the addition of water (20 mL) and then concentrated under reduced pressure to an oil. The oil was partitioned between ethyl acetate (100 mL) and 20% aqueous citric acid (100 mL). The organic phase was washed with water (2x100 mL), dried over sodium sulfate, concentrated under reduced pressure to an oil. The oil was redissolved in a minimum of dichloromethane (10 mL) and added with vigorous stirring to hexanes (100 mL) to give a precipitate. This was collected by filtration, washed with hexanes (3x50 mL) and dried (1 mm Hg, 25°C, 24 h) to 1.8 g (58%) of product as a white solid. H-NMR (DMSO-d6) d 2.4-2.65 (m, 6, $CH_2CH_2CO_2H$ and 2'-CH_2), 3.0-3.4 (m, 2, 5'and 5"-H), 3.74 (s, 6, O-CH_3), 4.18 (m, 1, 4'-H), 5.37 (m, 1, 3'-H), 6.30 (dd, 1, 1'-H), 6.81 (m, 4, arom.), 7.2-7.45 (m, 9, arom.), 7.92 (br s, 2, NH_2), 8.25 (s, 1, 8-H), 12.32 (br s, 1, CO_2H).

Example 21: Oligonucleotide synthesis and purification

Oligonucleotide synthesis

Oligonucleotides were synthesized on a Perseptive Biosystems Expedite 8901 Nucleic Acid Synthesis System. The following summarizes the 1-μmol scale synthesis protocol. Two synthesizers were used. One was set up to run the diester (P=O) protocol and the other to run the thioate (P=S) protocol. Trityl groups were removed with trichloroacetic acid (975 μL over one minute) followed by an acetonitrile wash.

Phosphodiester (P=O) protocol

All standard 2'-deoxy amidites (0.1M) were coupled twice per cycle (total coupling time was approximately 4 minutes). All 2'-modified amidites were dissolved in dry acetonitrile (100 mg of amidite/1 mL acetonitrile) to give approximately 0.08-0.1 M solutions. Total coupling time was approximately 6 minutes (105 μL of amidite delivered). 1-H-tetrazole in acetonitrile was used as the activating agent. Excess amidite was washed away with acetonitrile. (1S)-(+)-(10-camphorsulfonyl) oxaziridine (CSO, 1.0 g CSO/8.72 ml dry

acetonitrile) was used to oxidize (4 minute wait step) delivering approximately 375 µL of oxidizer.

Thioate (P=S) protocol

The same amounts of amidites and reagents were delivered to the columns using the thioate protocol, except that the amount of oxidizer, 3H-1,2-benzodithiole-3-one-1,1-dioxide (Beaucage reagent, 3.4 g Beaucage reagent/200 mL acetonitrile), was 225 µL (one minute wait step). In both protocols, unreacted functionalities were capped with a 50:50 mixture of tetrahyrdofuran/acetic anhydride and tetrahydrofuran/pyridine/1-methyl imidazole. Trityl yields were followed by the trityl monitor during the duration of the synthesis. The final DMT group was left intact.

10 µmole protocols

Approximately 300 mg CPG was used for each 10 µmole synthesis. For the diester (P=O) couplings, the following protocol was used: Trityl group removal was done using 7.5 mLs of trichloracetic acid. Coupling of both standard and novel amidites (0.8-0.1M) was achieved using a 23 minute coupling cycle with approximately 750 µL of amidite delivered. Approximately 2.7 mL of oxidizer (CSO) was delivered over 3 minutes. The capping was done over 80 seconds with 1.8 mLs of a 50:50 mixture of tetrahyrdofuran/acetic anhydride and tetrahydrofuran/pyridine/1-methyl imidazole. The thioate (P=S) couplings removed the trityl groups with 7.5 mLs of trichloroacetic acid. Couplings of standard 0.1M amidites were accomplished using 750 µL of solution over 6.6 minutes. The novel amidite couplings were done using 750µL of 0.08-0.1M solutions, delivered over 23 minutes. Approximately 2.7 mL of oxidizer was delivered over 3 minutes. The capping was done over 80 seconds with 1.8 mLs of a 50:50 mixture of tetrahyrdofuran/acetic anhydride and tetrahydrofuran/pyridine/1-methyl imidazole.

At 55°C, the 1 µmole syntheses were deprotected and cleaved from the CPG in 1-2 mL 28.0-30% ammonium hydroxide (NH_4OH) for approximately 16 hours. The 10µmole synthesis

CPG's was split into 2 vials for cleavage as described above. 5mg NH_4OH was added to each vial.

Oligonucleotide Purification

After the deprotection step, the samples were filtered from CPG using Gelman 0.45 um nylon acrodisc syringe filters. Excess NH_4OH was evaporated away in a Savant AS160 automatic speed vac. The crude yield was measured on a Hewlett Packard 8452A Diode Array Spectrophotometer at 260 nm. Crude samples were then analyzed by mass spectrometry (MS) on a Hewlett Packard electrospray mass spectrometer. Trityl-on oligos were purified by reverse phase preparative high performance liquid chromatography (HPLC). HPLC conditions were as follows: Waters 600E with 991 detector; Waters Delta Pak C4 column (7.8X300mm); Solvent A: 50 mM triethylammonium acetate (TEA-Ac), pH 7.0; B: 100% acetonitrile; 2.5 mL/min flow rate; the gradient: 5% B for first five minutes with linear increase in B to 80% during the next 55 minutes. Fractions containing the desired product were collected and the solvent was dried off in the speed vac. Oligos were detritylated in 80% acetic acid for approximately 60 minutes and lyophilized again. Free trityl and excess salt were removed by passing detritylated oligos through Sephadex G-25 (size exclusion chromatography) and collecting appropriate samples through a Pharmacia fraction collector. The solvent was again evaporated away in a speed vac. Purified oligos were then analyzed for purity by CGE, HPLC (flow rate: 1.5 mL/min; Waters Delta Pak C4 column, 3.9X300mm), and MS. The final yield was determined by spectrophotometer at 260 nm.

Oligonucleotide Analysis

Mass spectrometry

0.5 OD of oligo in an approximately 50 isopropanol:50 water mix (total volume of 100 mL including 2.5 mL 100 mM piperidine) was analyzed by mass spectrometry (Hewlett Packard 59987A Series II API 5989A electrospray mass spectrometer). The purified 0.5 OD sample was first desalted by osmosis on a Millipore filter (filter type VS with 0.025

mM pore size) for one hour. (Water with 10 drops of 28.0-30% NH_4OH were added to a petri dish. The 0.5 OD oligo sample was placed on the filter, floating on the water, for approximately one hour.) Purified yields were quantitated using a Hewlett Packard 8452A Diode Array Spectrophotometer at 260 nm.

^{31}P NMR analysis

^{31}P NMR analysis indicated the right number of P=O and P=S linkages. The P=S chemical shifts are dispersed over 2-3 ppm due to the diasteromeric nature of the P=S linkages.

Example 22: Oligonucleotide Sequences

Oligonucleotides synthesized with both 2-aminoadenosines and 5-methylcytidines are shown in Table 1. Several oligonucleotides were synthesized as gapmers havings a central region of 2'-deoxy residues flanked by regions of 2'-methoxyethoxy residues. Other oligonucleotides were synthesized as having all 2'-deoxy residues. All linkages in both sets of oligonucleotides were phosphorothioate linkages.

The oligonucleotides were characterized by mass spectrometry and HPLC analysis to verify the correct chemical structure. Results are shown in Table 1. All oligonucleotides synthesized gave the expected molecular weights.

Additional oligonucleotides are synthesized as described in Example 21. The structures of the oligonucleotides, and their physical characteristics, are shown in Table 2.

Table 1. Oligonucleotides containing 2-aminoadenosine and 5-methylcytidine

ISIS NO,	Nucleotide Sequence (5'-3')[1]	SEQ ID NO:	Target	Expected Mass (g/mol)	Observed Mass (g/mol)	HPLC Retn. Time (min)
17236	**A**TG**CA**TTCTGCCCC**CAA**GG**A**	1	Rat *C-raf*	7344	7344	--------
23107	**A**TG**CA**TTCTGCCCC**CAA**GG**A**	1	Rat *C-raf*	7362	7358	25.16[2]
32256	**A**TGC**A**TTCTGCCCCC**AA**GG**A**	1	Rat *C-raf*	7196	7196	26.07[3]
24681	TCCGTCATCGCTCCTCAGGG	2	Human *Ha-ras*	6492	6491	21.54[3]
24682	GCCCAAGCTGGCATCCGTCA	3	Human ICAM-1	6540	6539	22.64[3]
24683	GTTCTCGCTGGTGAGTTTCA	4	Human PKC-a	6521	6521	21.80[3]
24684	TCCCGCCTGTGACATGCATT	5	Human *raf* kinase	6492	6493	22.13[3]

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). Underlined cytidines are 5-methylcytidines; underlined adenosines are 2-aminoadenosines; all linkages are phosphorothioate linkages.

[2] HPLC Conditions: Waters 600E with 991 Detector; Waters DeltaPak C4 Column (3.9x300mm); Solvent A: 0.05M TEA-Ac; Solvent B: CH_3CN; Flow Rate: 1.5 mL/min.; Gradient: 5% B for first 10 min. with linear increase in B to 80% over the next 55 minutes.

[2]HPLC Conditions: Waters 600E with 991 Detector; Waters DeltaPak C4 Column (3.9x300mm); Solvent A: 0.05M TEA-Ac; Solvent B: CH_3CN; Flow Rate: 1.5 mL/min.; Gradient: 5% B for first 5 min. with linear increase in B to 60% over the next 55 minutes.

Table 2: Oligonucleotides Containing 2-NH_2-A and 5-Me-C with Alternating PS/PO Linkages, Fully Modified P=O Linkages, Fully Modified P=S Linkages, Gapmers, and Hemimers

SEQ ID NO:	Sequence (5'-3')[1]	Target
6	5'-$T_sC_oT_sG_oA_sG_oT_sA_oG_sC_oA_sG_oA_sG_oG_sA_oG_sC_oT_sC$-3'	Human ICAM-1
6	5'-$T_oC_oT_oG_oA_oG_oT_oA_oG_oC_oA_oG_oA_oG_oG_oA_oG_oC_oT_oC$-3'	Human ICAM-1
6	5'-$T_sC_sT_sG_sA_sG_sT_sA_sG_sC_sA_sG_sA_sG_sG_sA_sG_sC_sT_sC$-3'	Human ICAM-1
1	5'-$A_sT_oG_sC_oA_sT_oT_sC_sT_sG_sC_sC_sC_sC_oC_sA_oA_sG_oG_sA$-3'	Mouse C-*raf*
1	5'-$A_oT_oG_oC_oA_oT_oT_sC_sT_sG_sC_sC_sC_sC_oC_oA_oA_oG_oG_oA$-3'	Mouse C-*raf*
1	5'-$A_sT_sG_sC_sA_sT_sT_sC_sT_sG_sC_sC_sC_sC_sC_sA_sA_sG_sG_sA$-3'	Mouse C-*raf*
3	5'-$G_sC_sC_sC_sA_sA_sG_sC_sT_sG_sG_sC_oA_sT_oC_sC_oG_sT_oC_sA$-3'	Human ICAM-1
3	5'-$G_sC_sC_sC_sA_sA_sG_sC_sT_sG_sG_sC_oA_oT_oC_oC_oG_oT_oC_oA$-3'	Human ICAM-1
3	5'-$G_sC_sC_sC_sA_sA_sG_sC_sT_sG_sG_sC_oA_sT_sC_sC_sG_sT_sC_sA$-3'	Human ICAM-1

[1]All emboldened nucleosides are 2'-*O*-methoxyethyl (2'-O-CH_2-CH_2-O-CH_3), all C's are 5-methylcytosine residues, all A's are 2-aminoadenosine residues

Example 23: Effect of Base Substitutions on Melting Temperature

Oligonucleotides 26481 (SEQ ID NO. 2), 26482 (SEQ ID NO. 3), 26483 (SEQ ID NO. 4), and 26484 (SEQ ID NO. 5), were synthesized in three additional variants: with no base modifications (parent), with all 5-methylcytosines (no 2-aminoadenosines), and with all 2-aminoadenosines (no 5-methylcytosines).

Melting temperatures were measured as described in Freier, S. and Altmann, K.-H. (Nucl. Acids Res., 1997, 25, 4429-4443).

Results of Tm studies are shown in Table 3.

Table 3. Tm changes due to 2-amino-A and 5-Me-dC substitutions (RNA target)

SEQ ID NO.	ISIS NO:	Tm Parent	Tm with 2-NH_2-A			Tm with 5-Me-dC			Tm with 2-NH_2-A & 5-Me-dC		
			Isis #	Tm	# of A	Isis #	Tm	# of C	Isis #	Tm	# of mods
2	2302	61.7	27729	65.4	4	8737	66.7	8	24682	72.0	12
3	3521	53.5	27728	54.7	2	109749	--	4	24683	57.2	6
4	5132	60.6	27727	63.5	3	12075	66.2	7	24684	67.6	10
5	2503	62.5	27730	65.5	2	14464	66.9	8	24681	70.0	10

Example 24: ICAM-1 Expression

Oligonucleotide Treatment of HUVECs

HUVEC cells (Clonetics, San Diego, CA) were washed three times with OPTIMEM™ (Life Technologies, Inc., Rockville, MD) and prewarmed to 37°C. Oligonucleotides were premixed with 10 mg/ml LIPOFECTIN® (Life Technologies, Inc.) in OPTIMEM™, serially diluted to the desired concentrations, and applied to washed cells. Basal and untreated (no oligonucleotide) control cells were also treated with LIPOFECTIN®. Cells were incubated for 4 h at 37°C, at which time the medium was removed and replaced with standard growth medium with or without 5 mg/ml TNF-α (R&D Systems, Minneapolis, MN). Incubation at 37°C was continued until the indicated times.

Quantitation of ICAM-1 Protein Expression by Fluorescence-activated Cell Sorter

Cells were removed from plate surfaces by brief trypsinization with 0.25% trypsin in PBS. Trypsin activity was quenched with a solution of 2% bovine serum albumin and 0.2% sodium azide in PBS (+Mg/Ca). Cells were pelleted by centrifugation (1000 rpm, Beckman GPR centrifuge), resuspended in PBS, and stained with 3 μl of the ICAM-1 specific antibody, CD54-PE (Pharmingin, La Jolla, CA) per 10^5 cells. Antibodies were incubated with the cells for 30 min at 4°C in the dark, under gently agitation. Cells were washed by centrifugation procedures and then resuspended in 0.3 ml of FacsFlow buffer (Becton Dickinson, Franklin Lakes, NJ) with 0.5% formaldehyde (Polysciences, Inc., Warrington, PA). Expression of cell surface ICAM-1 was then determined by flow cytometry using a Becton Dickinson FACScan. Percentage of the control ICAM-1 expression was calculated as follows: [(oligonucleotide-treated ICAM-1 value) - (basal ICAM-1 value)/(non-treated ICAM-1 value) - (basal ICAM-1 value)]. (Baker, B., *et. al.*, *J. Biol. Chem.*, **1997** *272*, 11994-12000)

Tested oligonucleotides are shown in Table 4. Results are shown in Table 5. The oligonucleotide with 2-aminoadenosines and 5-methylcytosines was slightly more effective than the hemimers having a region of 2-methoxyethoxy nucleotides. A scrambled control of the parent oligonucleotide had little effect on ICAM-1 expression.

Table 4: Human ICAM-1 Antisense Oligonucleotides

Isis No.	SEQ ID NO:	Sequence (5'-3')[1]
15839	3	5'-$G_s\underline{C}_s\underline{C}_s\underline{C}_sA_sA_sG_s\underline{C}_sT_sG_sG_s\underline{C}_s\mathbf{A}_s\mathbf{T}_s\underline{\mathbf{C}}_s\underline{\mathbf{C}}_s\mathbf{G}_s\mathbf{T}_s\underline{\mathbf{C}}_s\mathbf{A}$-3'
16394	3	5'-$G_sC_sC_sC_sA_sA_sG_sC_sT_sG_sG_sC_o\mathbf{A}_o\mathbf{T}_o\underline{\mathbf{C}}_o\underline{\mathbf{C}}_o\mathbf{G}_o\mathbf{T}_o\underline{\mathbf{C}}_o\mathbf{A}$-3'
24862	3	5'-$G_s\underline{C}_s\underline{C}_s\underline{C}_s\underline{A}_s\underline{A}_sG_s\underline{C}_sT_sG_sG_s\underline{C}_s\underline{A}_sT_s\underline{C}_s\underline{C}_sG_sT_s\underline{C}_s\underline{A}$-3'
25303	3	5'-$G_s\underline{C}_s\underline{C}_s\underline{C}_sA_sA_sG_s\underline{C}_sT_sG_sG_s\underline{C}_o\mathbf{A}_s\mathbf{T}_o\underline{\mathbf{C}}_s\underline{\mathbf{C}}_o\mathbf{G}_s\mathbf{T}_o\underline{\mathbf{C}}_s\mathbf{A}$-3'

[1]All emboldened nucleosides are 2'-*O* -methoxyethyl (2'-O-CH_2-CH_2-O-CH_3), underlined C's are 5-methylcytosine residues, underlined A's are 2-aminoadenosine residues

TABLE 5

Inhibition of Human ICAM-1 RNA Expression in HUVEC Cells with Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	dose (mg/ml)	% protein Expression	% protein Inhibition
basal	---	---	---	---
induced	---	---	100.0%	---
25303	3	3	56.5%	43.5%
"	"	10	23.8%	76.2%
"	"	30	10.5%	89.5%
"	"	100	0.3%	99.7%
16394	3	3	55.5%	44.5%
"	"	10	26.9%	73.1%
"	"	30	13.6%	86.4%
"	"	100	3.4%	96.6%
24682	3	3	58.6%	41.4%
"	"	10	16.3%	83.7%
"	"	30	0.8%	99.2%
"	"	100	0.0%	100.0%
15839	3	3	55.4%	44.6%
"	"	10	28.1%	71.9%
"	"	30	6.3%	93.7%
"	"	100	0.0%	100.0%

Example 25: H-ras Expression

H-ras targeted antisense oligonucleotides were tested for the ability to specifically reduce H-ras mRNA in T-24 cells (ATCC, Manassas, Va.). T-24 cells were

routinely maintained in complete growth media, DMEM supplemented with 10% fetal calf serum and 100 units per milliliter penicillin and 100 micrograms per milliliter streptomycin (Life Technologies, Grand Island, NY) in a humidified incubator at 37°C. For antisense experiments T-24 cells were plated in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of $2x10^5$ cells per well in complete growth medium and incubated as above. Twenty-four hours after plating the growth media was aspirated and the monolayer was washed once with serum free media (OPTIMEM™, Life Technologies, Grand Island, NY). Oligonucleotides were formulated in serum free OPTIMEM™ and LIPOFECTIN® (Life Technologies, Grand Island, NY) at a constant ratio of 3 micrograms per milliliter LIPOFECTIN® per 100 nanomolar oligonucleotide. For oligonucleotide treatment two milliliters of formulated oligonucleotide was added to each well and the cells were incubated for four hours at 37°C. Following incubation the formulated oligonucleotide was aspirated from the monolayer, replaced with growth media, and incubated overnight. Twenty-four hours after treatment total RNA was prepared using RNAzol (TEL-TEST, Inc., Friendswood, TX.) following the manufacturer's protocol. RNA was fractionated through 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) following standard protocols (Sambrook *et al.*, Molecular Cloning a Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. **1989**). Nylon membranes were probed for H-ras (Oncogene Research Products, Cambridge, MA) using standard ^{32}P random priming labeling and hybridization protocols (Sambrook *et al.*, Molecular Cloning a Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. **1989**). Following hybridization, membranes were imaged using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the images quantified using Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA). Following image analysis, membranes were striped of H-ras probe and reprobed for G3PDH (Clontech, Palo Alto, CA) and analyzed as above. H-ras signal was normalized to G3PDH. The mean normalized percent control of triplicates and standard deviation for H-ras signal was calculated.

Tested oligonucleotides are shown in Table 6. Results are shown in Table 7. The oligonucleotide with 2-aminoadenosines and 5-methylcytosines was about three-fold more effective than a gapmer having a central region of 2'-deoxynucleotides flanked by

regions of 2'-methoxyethoxy nucleotides.

Table 6. Human H-ras Antisense Oligonucleotides

ISIS NO,	SEQ ID NO:	Nucleotide Sequence (5'-3')[1]
2503	2	TCCGTCATCGCTCCTCAGGG
13920	2	**TCC**GTCATCGCT**CCTC**AGGG
24681	2	TCCGTCATCGCTCCTCAGGG

[1] Emboldened residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). Underlined cytidines are 5-methylcytidines; underlined adenosines are 2-aminoadenosines; all linkages are phosphorothioate linkages.

TABLE 7

Inhibition of Human H-ras mRNA Expression in T-24 Cells with Antisense Oligonucleotides

ISIS No:	SEQ ID NO:	dose (mg/ml)	% protein Expression	% protein Inhibition
basal	---	---	100.0%	---
2503	2	33	97.2%	2.8%
"	"	100	63.9%	36.1%
"	"	300	42.5%	57.5%
13920	2	33	51.4%	48.6%
"	"	100	29.0%	71.0%
"	"	300	16.0%	84.0%
24681	2	33	33.0%	67.0%
"	"	100	16.0%	84.0%
"	"	300	13.1%	86.9%

WHAT IS CLAIMED IS:

1. An oligonucleotide comprising at least one 2-aminoadenosine nucleoside unit, at least one phosphorothioate internucleoside linkage, and at least one 5-substituted uridine or cytidine nucleoside unit

2. The oligonucleotide of claim 1 wherein said 5-substituent is bromo, fluoro, chloro or iodo.

3. The oligonucleotide of claim 1 wherein said 5 substituent is methyl.

4. The oligonucleotide of claim 1 comprising at least one 5-methylcytidine unit.

5. The oligonucleotide of claim 1 comprising at least one nucleoside unit having a 2'-O-modification.

6. The oligonucleotide of claim 5 wherein said 2'-O-modification is alkoxyalkyl, alkyl or dimethylaminooxyethyl.

7. The oligonucleotide of claim 1 comprising at least one 5- fluoro, bromo, chloro, iodo, or methyl cytidine or uridine unit and at least one 2'-O- alkoxyalkyl, alkyl or dimethylaminooxyethy - modified nucleoside unit.

8. The oligonucleotide of claim 1 having SEQ ID NO. 2, 3, 4, or 5.

9. A method of inhibiting expression of a gene comprising administering to cells or tissues the oligonucleotide of claim 1, said oligonucleotide having a sequence complementary to a portion of said gene.

10. A method of treating a disease or condition, associated with the expression of a gene, in an animal, comprising administering to said animal the oligonucleotide of claim 1, said oligonucleotide having a sequence complementary to said gene, immune stimulation in said animal being reduced thereby.

SEQUENCE LISTING

<110> Manoharan, Muthiah
Cook, Phillip Dan

<120> Oligonucleotides Incorporating Both 2-Aminoadenine and 5-Substituted Pyrimidines

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18415

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12P 19/34; C07H 21/00, 21/02, 21/04;A01N 43/04
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6,01.1, 325, 366, 375, 455; 536/23.1, 24.3, 24.5, 25.3; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, West, Sequence search.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,582,986A (MONIA et al.) 10 December 1996, col. 5, line 66-column 6, line 29.	1-8
Y	US 5,744,362A (MONIA et al.) 28 April 1998, col 1, line 21-col 2, line 48; col 8, line 44-col 14, line 8.	8
Y	PROSNYAK et al. Substitution of 2-Aminoadenine and 5-Methylcytosine for Adenine and Cytosine in Hybridization Probes Increases the Sensitivity of DNA Fingerprinting. Genomics 1994. Vol. 21, pages 490-494, see especially discussion, pages 492-493.	1-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other spec: l reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of parti-ular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 AUGUST 2000	18 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Jane Zara Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18415

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BUTTINELLI et al. The exocyclic groups of DNA modulate the affinity and positioning of the histone octamer. Proc. Natl. Acad. Sci. July 1998. Vol. 95, pages 8544-8549, figures 1 and 3, page 8545,	1-8
Y	LAMM et al. Antisense probes containing 2-aminoadenosine allow efficient depletion of U5 snRNP from HeLa splicing extracts. Nucleic Acids Res., 1991. Vol. 19, No. 12, pages 3193-3198, entire text, especially figure1, page 3194 and results, left column, page 3195.	1-8
Y	SANGHVI et al. Antisense oligodeoxynucleotides: synthesis, biophysical and biological evaluation of oligodeoxynucleotides containing modified pyrimidines. Nuc. Acids Res. 1993. vol. 21, No. 14, pages 3197-3203, see expecially Table 3, page 3200 and section entitled "Nucleolytic degradation", page 3199.	2, 5-7
Y	BARABINO et al. Antisense probes targeted to an internal domain in U2 snRNP specifically inhibit the second step of pre-mRNA splicing. Nucl. Acids Res., 1992, Vol. 20, No. 17, pages 4457-4464, abstract; paragraphs 2-4 of the introduction, pagc 4457-8 and references cited therein; figure 1, page 4459.	5-7
Y	US 5,801,154 A (BARACCHINI et al.) 01 September 1998, col. 6, line 58-col 7, line 38.	5-7
Y	US 5,883,082 A (BENNETT et al.) 16 March 1999, abstract; col 3, lines 30-35, col 19, line 35-col 20, line 29.	8
Y	US 5,703,054 A (BENNETT et al.) 30 December 1997, col 9, line 17-col 11, line 33; col 19, line 35-col 20, line 29.	8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18415

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6,01.1, 325, 366, 375, 455; 536/23.1, 24.3, 24.5, 25.3; 514/44