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(54) Title: AMINOOXY-MODIFIED OLIGONUCLEOTIDES

(57) Abstract

Nucleotide compositions containing aminooxy moieties are provided. In accordance with preferred embodiments, oligonucleotides and oligonucleotide analogs are provided which are specifically hybridizable with a selected sequence of RNA or DNA wherein at least one of the nucleoside moieties of the oligonucleotide is modified to include an aminooxy moiety.

AMINOOXY-MODIFIED OLIGONUCLEOTIDES

RELATED APPLICATION DATA

This patent application claims priority benefit of U.S. Provisional Application Serial No. 60/037,143, filed on February 14, 1997, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

This invention is directed to aminooxy modified oligonucleotides. Such oligonucleotides are useful as therapeutics, diagnostics, and research reagents. The invention is further directed to aminooxy nucleotides, nucleoside and nucleoside surrogates that are useful as precursors for the preparation of oligonucleotides. In certain embodiments of the invention, the inclusion of one or more aminooxy moieties of the invention in an oligonucleotide provides, *inter alia*, for improved binding of the oligonucleotides to a complementary strand. In further embodiments of the invention, the inclusion of one or more aminooxy moieties provides one or more conjugation sites useful for the conjugation of various useful ligands to the oligonucleotides. Such ligands include, for example, reporter groups and groups for modifying uptake, distribution or other pharmacodynamic properties.

BACKGROUND OF THE INVENTION

It has been recognized that oligonucleotides can be used to modulate mRNA expression by a mechanism that involves the complementary hybridization of relatively short oligonucleotides to mRNA such that the normal, essential functions of these intracellular nucleic acids are disrupted. Hybridization is the sequence-specific base pair hydrogen bonding of an oligonucleotide to a complementary RNA or DNA.

For use in diagnostics and as research reagents and as well in therapeutics, the ability of an oligonucleotide to bind to a specific DNA or RNA with fidelity is an important factor. The relative ability of an oligonucleotide to bind to complementary nucleic acids is compared by determining the melting temperature of a particular hybridization complex. The melting temperature (T_m), a characteristic physical property of double helices, is the temperature (in °C) at which 50% helical versus coil (unhybridized) forms are present. T_m is measured by using UV spectroscopy to determine the formation and breakdown (melting) of hybridization. Base stacking, which occurs during hybridization, is accompanied by a reduction in UV absorption (hypochromicity). Consequently, a reduction in UV absorption indicates a higher T_m. The higher the T_m, the greater the strength of the binding of the nucleic acid strands. Therefore, oligonucleotides modified to hybridize with appropriate strength and fidelity to its targeted RNA (or DNA) are greatly desired for use as research reagents, diagnostic agents and as oligonucleotide therapeutics.

Various substitutions have been introduced in the base and sugar moieties of the nucleosides of oligonucleotides. The inclusion of certain of these substitutions has resulted in improvements in the resulting oligonucleotide. One such useful improvement is an increase in the nuclease resistance of the oligonucleotides by the introduction of 2'-substituents such as halo, alkoxy and allyloxy groups.

Ikehara *et al.* (*European J. Biochem.*, **1984**, *139*, 447) have reported the synthesis of a mixed octamer containing one 2'-deoxy-2'-fluoroguanosine residue or one

2'-deoxy-2'-fluoroadenine residue. Guschlbauer and Jankowski (*Nucleic Acids Res*, 1980, *8*, 1421) have shown that the contribution of the 3'-endo increases with increasing electronegativity of the 2'-substituent. Thus, 2'-deoxy-2'-fluorouridine contains 85% of the C3'-endo conformer.

Furthermore, evidence has been presented which indicates that 2'-substituted-2'-deoxyadenosine polynucleotides resemble double-stranded RNA rather than DNA. Ikehara *et al.* (*Nucleic Acids Res.*, 1978, *5*, 3315) have shown that a 2'-fluoro substituent in poly A, poly I, or poly C duplexed to its complement is significantly more stable than the ribonucleotide or deoxyribonucleotide poly duplex as determined by standard melting assays. Ikehara *et al.* (*Nucleic Acids Res.*, 1978, *4*, 4249) have shown that a 2'-chloro or bromo substituent in poly(2'-deoxyadenylic acid) provides nuclease resistance. Eckstein *et al.* (*Biochemistry*, 1972, *11*, 4336) have reported that poly(2'-chloro-2'-deoxy-uridylic acid) and poly(2'-chloro-2'-deoxycytidylic acid) are resistant to various nucleases. Inoue *et al.* (*Nucleic Acids Res.*, 1987, *15*, 6131) have described the synthesis of mixed oligonucleotide sequences containing 2'-OMe substituents on every nucleotide. The mixed 2'-OMe-substituted oligonucleotide hybridized to its RNA complement as strongly as the RNA-RNA duplex which is significantly stronger than the same sequence RNA-DNA heteroduplex (T_ms, 49.0 and 50.1 versus 33.0 degrees for nonamers). Shibahara *et al.* (*Nucleic Acids Res.*, 1987, *17*, 239) have reported the synthesis of mixed oligonucleotides containing 2'-OMe substituents on every nucleotide. The mixed 2'-OMe-substituted oligonucleotides were designed to inhibit HIV replication.

It is believed that the composite of the hydroxyl group's steric effect, its hydrogen bonding capabilities, and its electronegativity versus the properties of the hydrogen atom is responsible for the gross structural difference between RNA and DNA. Thermal melting studies indicate that the order of duplex stability (hybridization) of 2'-methoxy

oligonucleotides is in the order of RNA-RNA > RNA-DNA > DNA-DNA.

U.S. Patent 5,013,830, issued May 7, 1991, discloses mixed oligonucleotides comprising an RNA portion, bearing 2'-O-alkyl substituents, conjugated to a DNA portion via a phosphodiester linkage. However, being phosphodiesters, these oligonucleotides are susceptible to nuclease cleavage.

European Patent application 339,842, filed April 13, 1989, discloses 2'-O-substituted phosphorothioate oligonucleotides, including 2'-O-methylribooligonucleotide phosphorothioate derivatives. This application also discloses 2'-O-methyl phosphodiester oligonucleotides which lack nuclease resistance.

European Patent application 260,032, filed August 27, 1987, discloses oligonucleotides having 2'-O-methyl substituents on the sugar moiety. This application also makes mention of other 2'-O-alkyl substituents, such as ethyl, propyl and butyl groups.

International Publication Number WO 91/06556, published May 16, 1991, and United States patent 5,466,786 discloses oligomers derivatized at the 2' position with substituents, which are stable to nuclease activity. Specific 2'-O-substituents which were incorporated into oligonucleotides include ethoxycarbonylmethyl (ester form), and its acid, amide and substituted amide forms.

European Patent application 399,330, filed May 15, 1990, discloses nucleotides having 2'-O-alkyl substituents.

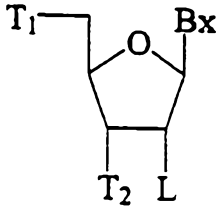
International Publication Number WO 91/15499, published October 17, 1991, discloses oligonucleotides bearing 2'-O-alkyl, -alkenyl and -alkynyl substituents.

Martin, P., *Helvetica Chimica Acta*, *78*, 1995, 486-504, discloses certain nucleosides and oligonucleotides prepared therefrom that include 2'-methoxyethoxy, 2'-methoxy(tris-ethoxy) and other substituents. Oligonucleotides containing nucleoside substituted with either the 2'-methoxyethoxy and 2'-methoxy(tris-ethoxy) substituents exhibited improved hybridization as judged by increase in Tm.

It has been recognized that oligonucleotides having improved fidelity hybridization are of great importance in the development of oligonucleotide useful as research reagents, diagnostic agents and therapeutic agents.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, aminooxy containing compositions that modulate the activity of DNA and RNA are provided. Preferred compositions include compounds of the structure:

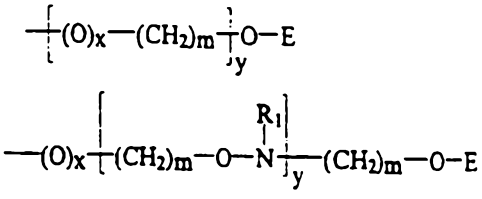


wherein:

B_X is purine or pyrimidine heterocyclic base;

T_1 and T_2, independently, are OH, a hydroxyl protecting group, an activated phosphate group, a nucleotide, a nucleoside, or an oligonucleotide; and

L has one of the structures;



wherein

m is from 1 to 10;

y is from 1 to 10;

x is 1;

E is $N(R_1)(R_2)$ or $N=C(R_1)(R_2)$; and

each R_1 and R_2 is, independently, H, C-C_{10} alkyl, a nitrogen protecting group, or R_1 and R_2, together, are a nitrogen protecting group or wherein R_1 and R_2 are joined in a ring structure that can

AUSTRALIAN PATENT OFFICE

include at least one heteroatom selected from N and O.

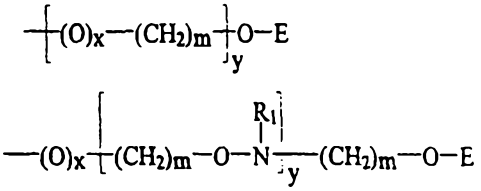
Bx preferably is adenine, guanine, hypoxanthine, uracil, thymine, cytosine, 2-aminoadenine or 5-methyl cytosine. R_1 and R_2 can be a ring structure such as imidazole, piperidine, morpholine or a substituted piperazine (*e.g.*, a piperazine substituted with a C_1-C_{12} alkyl). Both of T_1 and T_2 can be oligonucleotides or one of them can be an oligonucleotide and the other can be a hydroxyl protecting group.

In certain embodiments, the compositions of the invention include compounds of the structure:

Q-L

wherein

L has one of the structures:



wherein

m is from 0 to 10;

y is from 1 to 10;

E is $N(R_1)(R_2)$ or $N=C(R_1)(R_2)$;

each R_1 and R_2 is, independently, H, C_1-C_{10} alkyl, a nitrogen protecting group, or R_1 and R_2, together, are a nitrogen protecting group or wherein R_1 and R_2 are joined in a ring structure that can include at least one heteroatom selected from N and O;

and X and Q are selected such that:

when X is 1, then Q is:

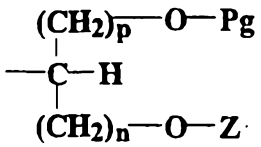
a nucleoside substituted with said L group at a 2', 3' or 5' position; or

a 3'-phosphitylated nucleoside substituted with said L group at a 2' or 5' position; or

a 2'-phosphitylated nucleoside substituted with said L group at a 3' or 5' position; or

an oligonucleotide that includes a nucleotide substituted at a 2', 3' or 5' position with said L group; or

when X is 0, then Q is a compound of the structure:



where

n and p are, independently, from 0 to 10 with the sum of n and p being greater than 2 and less then 11;

Pg is a hydroxyl protecting group (*e.g.*, dimethoxytrityl); and

Z is a solid support or a protected and activated phosphorus moiety (*e.g.*, a cyanoethoxy-N,N-diisopropyl phosphoramidite group).

Preferably, p is 1 and n is 4.

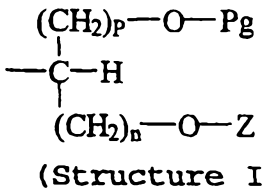
In a preferred group of compounds, y is 1 to 4. In an even more preferred groups of compounds y is 1.

In a preferred group of compounds, m is 1 to 6. In an even more preferred groups of compounds m is 2.

In a preferred group of compounds, R_1 and R_2 are H or alkyl. In a further group of compounds R_1 and R_2 are a nitrogen protecting group. Preferred nitrogen protecting groups include phthalimido-N-oxy and formaloximyl.

In a preferred group of compounds Q is a nucleoside, nucleotide or a nucleoside that is incorporated in an oligonucleotide. In a more preferred group of compounds Q is a 2' or 3' substituted nucleoside that is incorporated in an oligonucleotide.

In a preferred group of compounds Q is of the structure:



(Structure I)

where Pg and Z are as defined above. In a further preferred group of compounds, Z is a solid support. In a further preferred group of compounds, Z is a protected and activate phosphorous atom. In an even more preferred group of compounds, Z is a protected phosphoramidite moiety.

Preferred compositions of the invention include oligonucleotides modified to include one or more of the above Q-L compounds. Thus these compositions comprised oligonucleotides having one or more aminooxy modified nucleosides of the invention or oligonucleotides modified to include one or more nucleoside surrogates of the formula Q-L where Q is Structure I above. These oligonucleotides are specifically hybridizable with preselected nucleotide sequences of single-stranded or double-stranded target DNA or RNA. The oligonucleotides recognize and form double strands with single-stranded DNA and RNA.

The modified oligonucleotides of the present invention consist of a single strand of nucleic acid bases linked together through linking groups. The oligonucleotides of this invention may range in length from about 5 to about 50 nucleic acid bases. However, in accordance with certain preferred embodiments of this invention, a sequence of about 12 to 25 bases in length is desired.

The preferred individual nucleotides of the oligonucleotides of the present invention can be connected via phosphorus linkages. Preferred phosphorous linkages include phosphodiester, phosphorothioate and phosphorodithioate linkages, with phosphodiester and phosphorothioate linkages being particularly preferred.

Preferred nucleobases of the invention include adenine, guanine, cytosine, uracil, thymine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, other aza and deaza thymidines, other aza and deaza cytosines, other aza and deaza adenines, other aza and deaza guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

In accordance with one embodiment of this invention at least one of the nucleosides of an oligonucleotide is modified. Preferred sites for modification of the nucleoside components are at the 2', 3' or 5' positions of the nucleoside. The modification comprises one that introduces an aminooxy moiety on to one or more nucleosides. In accordance with a further embodiment of the invention, at least one nucleoside surrogate having an aminooxy moiety is utilized to modify the oligonucleotide. The aminooxy modified nucleoside or aminooxy containing nucleoside surrogate is incorporated into the oligonucleotide using standard synthetic methods for preparing oligonucleotides resulting in incorporation of the aminooxy moiety in the oligonucleotide.

A preferred modification comprises oligonucleotides that include one or more nucleoside units that are modified to include 2', 3' or 5'-O-aminooxyalkyl, 2', 3' or 5'-O-alkylaminooxyalky or 2', 3' or 5'-O-dialkylaminooxyalkyl modification. Further preferred modifications comprise nucleoside surrogates comprising alkyl units that have first and second hydroxy functionalities thereon for incorporation into an oligonucleotides and that have a side chain having an aminooxy moiety thereon for use in linking conjugate groups to the oligonucleotide via conjugation at the aminooxy moiety. Such conjugation is effected by suitable alkylation or

acylation of the nitrogen atom of the aminooxy moiety. The vitamin A family of compounds can be attached to oligonucleotides via acid or alcohol functionalities found in the various family members. For example, conjugation of an N-hydroxy succinimide ester of an acid moiety of retinoic acid to an amine function on a linker pendant to an oligonucleotide results in linkage of the vitamin A compound to the oligonucleotide via an amide bond. Also, retinol has been converted to its phosphoramidite, which is useful for 5' conjugation.

Suitable for selection as conjugate groups or ligands are: a steroid molecule, a reporter molecule, a lipophilic molecule, a reporter enzyme, a peptide, a protein (*i.e.*, a substituent consisting essentially of same), or a glycol or glycol like linker. For the purposes of this invention the terms "reporter molecule" and "reporter enzyme" are inclusive of those molecules or enzymes that have physical or chemical properties that allow them to be identified in gels, fluids, whole cellular systems, broken cellular systems and the like utilizing physical properties such as spectroscopy, radioactivity, colorimetric assays, fluorescence, and specific binding. Steroids include those chemical compounds that contain a perhydro-1,2-cyclopentanophenanthrene ring system. Proteins and peptides are utilized in their usual sense as polymers of amino acids. Normally peptides comprise such polymers that contain a smaller number of amino acids per unit molecule than do the proteins. Lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, esters, alcohols and other lipid molecules, substituted aromatic groups such as dinitrophenyl groups, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

Particularly useful as steroid molecules are the bile acids including cholic acid, deoxycholic acid and dehydrocholic acid; steroids including cortisone, digoxigenin, testosterone

and cholesterol and even cationic steroids such as cortisone having a trimethylaminomethyl hydrazide group attached via a double bond at the 3 position of the cortisone rings. Particularly useful as reporter molecules are biotin, dinitrophenyl, and fluorescein dyes. Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids, waxes, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes. Particularly useful as reporter enzymes are alkaline phosphatase and horseradish peroxidase. Particularly useful as peptides and proteins are sequence-specific peptides and proteins including phosphodiesterase, peroxidase, phosphatase and nuclease proteins. Such peptides and proteins include SV40 peptide, RNaseA, RNase H and Staphylococcal nuclease. Particularly useful as terpenoids are vitamin A, retinoic acid, retinal and dehydroretinol. Other conjugate ligands are described in United States patent 5,578,718, commonly assigned with this application and herein incorporated by reference.

The present invention also includes oligonucleotides formed from a plurality of linked nucleosides including 2'-deoxy-*erythro*-pentofuranosyl-ß-nucleosides. These nucleosides are connected by charged phosphorus linkages in a sequence that is specifically hybridizable with a complementary target nucleic acid. The sequence of linked nucleosides is divided into at least two subsequences. The first subsequence includes nucleosides, having 2'-aminooxyalkyl substituents, linked by charged 3'-5' phosphorous linkages. The second subsequence consists of 2'-deoxy-*erythro*-pentofuranosyl-ß-nucleosides linked by charged 3'-5' phosphorous linkages bearing a negative charge at physiological pH. In further preferred embodiments there exists a third subsequence whose nucleosides are selected from those selectable for the first subsequence. In preferred embodiments the second subsequence is positioned between the first and third subsequences. Such oligonucleotides of the present invention are also referred to as "chimeric" or "gapped" oligonucleotides, or "chimeras."

The resulting novel oligonucleotides of the invention will have increased resistant to nuclease degradation and will exhibit hybridization properties of higher quality relative to wild-type DNA-DNA and RNA-DNA duplexes and phosphorus-modified oligonucleotide duplexes containing methylphosphonates, phophoramidates and phosphate triesters or they will have nucleoside surrogate units incorporated therein having aminooxy sites on the surrogate unit for linking conjugate groups to the oligonucleotide via the aminooxy moiety.

The invention is also directed to methods for modulating the production of a protein by an organism comprising contacting the organism with a composition formulated in accordance with the foregoing considerations. It is preferred that the RNA or DNA portion which is to be modulated be preselected to comprise that portion of DNA or RNA which codes for the protein whose formation is to be modulated. Therefore, the oligonucleotide to be employed is designed to be specifically hybridizable to the preselected portion of target DNA or RNA.

This invention is also directed to methods of treating an organism having a disease characterized by the undesired production of a protein. This method comprises contacting the organism with a composition in accordance with the foregoing considerations. The composition is preferably one which is designed to specifically bind with mRNA which codes for the protein whose production is to be inhibited.

The invention further is directed to diagnostic methods for detecting the presence or absence of abnormal RNA molecules, or abnormal or inappropriate expression of normal RNA molecules in organisms or cells.

The invention is also directed to methods for the selective binding of RNA for use as research reagents and diagnostic agents. Such selective and strong binding is accomplished by interacting such RNA or DNA with oligonucleotides of the invention which display elevated fidelity of hybridization.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a synthesis of certain intermediates of the invention.

Figure 2 shows a synthesis of 5-methyluridine DMT-phosphoroamidite having a protected aminooxyethyl group at the 2'-O position.

Figure 3 shows a synthesis of certain intermediates of the invention.

Figure 4 shows a synthesis of adenosine DMT-phosphoroamidite having a protected aminooxyethoxy group at the 2' position.

Figure 5 shows a synthesis of certain intermediates of the invention.

Figure 6 shows a synthesis of cytidine DMT-phosphoroamidite having a protected aminooxyethoxy group at the 2' position.

Figure 7 shows a synthesis of certain intermediates of the invention.

Figure 8 shows a synthesis of guanidine DMT-phosphoroamidite having a protected aminooxyethoxy group at the 2' position.

Figure 9 shows a synthesis of some intermediates and monomers of the invention.

Figure 10 shows a linking of compounds of the invention to CPG.

Figure 11 shows a synthesis of intermediates and monomers of the invention.

Figure 12 shows a synthesis of intermediates and monomers of the invention.

Figure 13 shows a graph of % full length oligonucleotide versus time in minutes pertaining to effects of nuclease action on oligonucleotides.

Figure 14 shows a graph of % full length oligonucleotide versus time in minutes pertaining to effects of nuclease action on oligonucleotides.

Figure 15 shows a graph of % full length oligonucleotide versus time in minutes pertaining to effects of nuclease action on oligonucleotides.

Figure 16 shows a synthesis of intermediates and monomers of the invention.

Figure 17 shows a synthesis of intermediates and monomers of the invention.

Figure 18 shows a synthesis of intermediates and monomers of the invention.

Figure 19 shows a synthesis of intermediates and monomers of the invention.

Figure 20 shows a synthesis of intermediates and monomers of the invention.

Figure 21 shows a synthesis of intermediates and monomers of the invention.

Figure 22 shows a synthesis of intermediates and monomers of the invention.

Figure 23 shows a synthesis of intermediates and monomers of the invention.

Figure 24 shows a synthesis of intermediates and monomers of the invention.

Figure 25 shows a synthesis of intermediates and monomers of the invention.

Figure 26 shows a synthesis of intermediates and monomers of the invention.

Figure 27 shows a synthesis of intermediates and monomers of the invention.

Figure 28 shows a synthesis of intermediates and monomers of the invention.

Figure 29 shows a synthesis of intermediates and monomers of the invention.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Compositions useful for identification or quantification of an RNA or DNA or for modulating the activity of an RNA or DNA molecule in accordance with this invention generally comprise a sugar-modified oligonucleotide which is

specifically hybridizable with a preselected nucleotide sequence of a single-stranded or double-stranded target DNA or RNA molecule. It is generally desirable to select a sequence of DNA or RNA which is involved in the production of a protein whose synthesis is ultimately to be modulated or inhibited in its entirety or to select a sequence of RNA or DNA whose presence, absence or specific amount is to be determined in a diagnostic test.

The oligonucleotides of the invention are conveniently synthesized using solid phase synthesis of known methodology, and is designed to be complementary to or specifically hybridizable with the preselected nucleotide sequence of the target RNA or DNA. Nucleic acid synthesizers are commercially available and their use is understood by persons of ordinary skill in the art as being effective in generating any desired oligonucleotide of reasonable length.

The oligonucleotides of the invention also include those that comprise nucleosides connected by charged linkages, and whose sequences are divided into at least two subsequences. The first subsequence includes 2'-aminooxyalkyl substituted-nucleosides linked by a first type of linkage. The second subsequence includes nucleosides linked by a second type of linkage. In a preferred embodiment there exists a third subsequence whose nucleosides are selected from those selectable for the first subsequence, and the second subsequence is positioned between the first and the third subsequences. Such oligonucleotides of the invention are known as "chimeras," or "chimeric" or "gapped" oligonucleotides.

In the context of this invention, the term "oligonucleotide" refers to a plurality of nucleotides joined together in a specific sequence from naturally and non-naturally occurring nucleobases. Preferred nucleobases of the invention are joined through a sugar moiety via phosphorus linkages, and include adenine, guanine, adenine, cytosine, uracil, thymine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine,

pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, other aza and deaza thymidines, other aza and deaza cytosines, other aza and deaza adenines, other aza and deaza guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine. The sugar moiety may be deoxyribose or ribose. The oligonucleotides of the invention may also comprise modified nucleobases or nucleobases having other modifications consistent with the spirit of this invention, and in particular modifications that increase their nuclease resistance in order to facilitate their use as therapeutic, diagnostic or research reagents.

The oligonucleotides of the present invention are about 5 to about 50 bases in length. It is more preferred that the oligonucleotides of the invention have from 8 to about 40 bases, and even more preferred that from about 12 to about 25 bases be employed.

It is desired that the oligonucleotides of the invention be adapted to be specifically hybridizable with the nucleotide sequence of the target RNA or DNA selected for modulation. Oligonucleotides particularly suited for the practice of one or more embodiments of the present invention comprise 2', 3', or 5'-sugar modified oligonucleotides wherein the 2', 3' or 5' position of the pentofuranosyl moieties of the nucleoside is modified to include an aminooxy moiety. For example, the oligonucleotides are modified to contain substitutions including but not limited incorporation of one or more nucleoside units modified as 2', 3' or 5'-O-aminooxyalkyl, 2', 3' or 5'-O-alkylaminooxyalkyl, 2', 3' or 5'-O-dialkylaminooxyethyl; or one or more nucleoside surrogate groups that include an alkyl group having one or two hydroxyl groups and an aminooxy group thereon; and protected, blocked or precursor forms of the above including phthalimido-N-oxyalkyl and formaloximylalkyl substitutions. The modified nucleosides or surrogate nucleosides can be positioned

internally in the oligonucleotide via linking in the oligonucleotide backbone or they can be located on one or both of the 3′ and 5′ terminal ends of the oligonucleotide.

The nucleoside surrogate can include appropriate activated phosphorous atoms in P^{III} or P^{V} valance states for incorporation into an oligonucleotide. Such activated phosphorous atoms include phosphoramidite, H phosphonates and triesters as are known in the art. The nucleoside surrogates can also include appropriate hydroxyl blocking groups including but not limited to dimethoxytrityl, trimethoxytrityl, monomethoxytrityl and trityl blocking groups and other blocking groups as are known in the art.

In positioning one of the nucleoside surrogate groups of the invention in an oligonucleotide, an appropriate blocked and activated nucleoside surrogate is incorporated in the oligonucleotides in the standard manner for incorporation of a normal blocked and active standard nucleotide. As for instance, an nucleoside surrogate is selected that has its aminooxy moiety protected utilizing a phthalimido protecting group. One of the hydroxyl groups of the surrogate molecule is protected utilizing a dimethoxytrityl protecting group (a DMT protecting group) and the other hydroxyl group, the second hydroxyl group, is present as an cyanoethoxy diisopropyl phosphoramidite moiety. The surrogate unit is added to the growing oligonucleotide by treating with the normal activating agents, as is known is the art, to react the phosphoramidite moiety with the growing oligonucleotide. This is followed by removal of the DMT group in the standard manner, as is known in the art, and continuation of elongation of the oligonucleotide with normal nucleotide amidite units as is standard in the art. If the nucleoside surrogate unit is an intermediate units utilized during synthesis of the oligonucleotide, the surrogate nucleoside is positioned in the interior of the oligonucleotide. If the nucleoside surrogate unit is the last unit linked to the oligonucleotide, the nucleoside surrogate unit will form the 5′ most terminal moiety of the oligonucleotide.

Other nucleoside surrogate units can include linkage of the second hydroxyl group to a solid support, in the same manner as is utilized for linkage of conventional nucleosides to solid supports. Upon cleavage of the oligonucleotide from the solid support, the nucleoside surrogate unit will form the 3' most terminal moiety of the oligonucleotide.

In each such oligonucleotide, upon cleavage of the oligonucleotide from its solid support, the phthalimido blocking group is removed to generate the aminooxy moiety on the oligonucleotide. The amino functionality of this aminooxy moiety can be further reacted with appropriate ligands via alkylation or acylation reactions to joint the ligand to the oligonucleotide as a conjugate group. Various conjugate groups, as are known in the art, can be conjugated to the oligonucleotide in this manner.

In the substituted nucleosides of the invention, each alkyl is a straight or branched chain of C_1 to C_{10}. For utilization in a 2' 3' or 5'-O-substituted nucleoside of the invention, a more preferred alkyl group is C_1-C_4 alkyl with C_2 alkyl being the most preferred.

For nucleoside surrogates of the invention, the overall length of the alkyl group will be selected to be less than 11 with the aminooxy group positioned between the ends of the alkyl group. In certain preferred nucleoside surrogates of the invention, it is preferred to position the aminooxy group with at least two methylene groups between it and either of the hydroxyl groups of the nucleoside surrogate. This can be accomplished by any combination of methylene units in either the alkyl backbone or on the aminooxy side chain. As so positioned the oxygen atom of the aminooxy moiety and the oxygen atoms of the hydroxyl groups do not form acetal type structures. In other embodiments the aminooxy moiety is positioned with only one methylene group between it and one or the other of the hydroxyl groups forming an acetal type structure.

In substituted nucleosides of the invention, a first preferred group of substituents include 2'-aminoxyalkyl

substituents. A further preferred group of substituents include alkylated aminooxyalkyl include dialkylaminooxyalkyl and monoalkylaminoalkyl, e.g., dimethylaminooxyethyl and ethylaminooxyethyl. An additional preferred group of substituents include precursor or blocked forms of these 2'-O-aminooxyalkyl substituents include phthalimido and formaldehyde adducts, i.e., phthalimido-N-oxy and formaloximyl groups.

In certain preferred embodiments of the present invention, the individual nucleosides of the oligonucleotides of the invention are connected via phosphorus linkages. Preferred phosphorus linkages include phosphodiester, phosphorothioate and phosphorodithioate linkages. In one preferred embodiment of this invention, nuclease resistance is conferred on the oligonucleotides by utilizing phosphorothioate internucleoside linkages.

In further embodiments of the invention, nucleosides can be joined via linkages that substitute for the internucleoside phosphate linkage e.g. substitute internucleoside linkages. Macromolecules of this type have been identified as oligonucleosides. The term "oligonucleoside" thus refers to a plurality of nucleoside units joined by non-phosphorus linkages.

Oligonucleotides and oligonucleosides can be joined to give a chimeric oligomeric compound. In addition to the naturally occurring phosphodiester linking group, phosphorus and non-phosphorus containing linking groups that can be used to prepare oligonucleotides, oligonucleosides and oligomeric chimeric compounds of the invention are well documented in the prior art and include without limitation the following:

<u>phosphorus containing linkages</u>

phosphorodithioate (-O-P(S)(S)-O-);

phosphorothioate (-O-P(S)(O)-O-);

phosphoramidate (-O-P(O)(NJ)-O-);

phosphonate (-O-P(J)(O)-O-);

phosphotriesters (-O-P(O J)(O)-O-);

phophosphoramidate (-O-P(O)(NJ)-S-);

thionoalkylphosphonate (-O-P(S)(J)-O-);
thionoalkylphosphotriester (-O-P(O)(OJ)-S-);
boranophosphate (-R^5-P(O)(O)-J-);
non-phosphorus containing linkages
thiodiester (-O-C(O)-S-);
thionocarbamate (-O-C(O)(NJ)-S-);
siloxane (-O-Si(J)$_2$-O-);
carbamate (-O-C(O)-NH- and -NH-C(O)-O-)
sulfamate (-O-S(O)(O)-N- and -N-S(O)(O)-N-;
morpholino sulfamide (-O-S(O)(N(morpholino)-);
sulfonamide (-O-SO_2-NH-);
sulfide (-CH_2-S-CH_2-);
sulfonate (-O-SO_2-CH_2-);
N,N'-dimethylhydrazine (-CH_2-N(CH_3)-N(CH_3)-);
thioformacetal (-S-CH_2-O-);
formacetal (-O-CH_2-O-);
thioketal (-S-C(J)$_2$-O-); and
ketal (-O-C(J)$_2$-O-);
amine (-NH-CH_2-CH_2-);
hydroxylamine (-CH_2-N(J)-O-);
hydroxylimine (-CH=N-O-); and
hydrazinyl (-CH_2-N(H)-N(H)-).

Wherein J denotes a substituent group which is commonly hydrogen or an alkyl group or a more complicated group that varies from one type of linkage to another.

In addition to linking groups as described above that involve the modification or substitution of the -O-P-O- atoms of a naturally occurring linkage, included within the scope of the present invention are linking groups that include modification of the 5'-methylene group as well as one or more of the -O-P-O- atoms. Linkages of this type are well documented in the prior art and include without limitation the following:

amides (-CH_2-CH_2-N(H)-C(O)) and -CH_2-O-N=CH-;and
alkylphosphorus (-C(J)$_2$-P(=O)(OJ)-C(J)$_2$-C(J)$_2$-).

wherein J is as described above.

Synthetic schemes for the synthesis of the substitute internucleoside linkages described above are disclosed in:

WO 91/08213; WO 90/15065; WO 91/15500; WO 92/20822; WO 92/20823; WO 91/15500; WO 89/12060; EP 216860; US 92/04294; US 90/03138; US 91/06855; US 92/03385; US 91/03680; U.S. Patent Nos. 07/990,848; 07,892,902; 07/806,710; 07/763,130; 07/690,786; 5,466,677; 5,034,506; 5,124,047; 5,278,302; 5,321,131; 5,519,126; 4,469,863; 5,455,233; 5,214,134; 5,470,967; 5,434,257; Stirchak, E.P., et al., *Nucleic Acid Res.*, **1989**, *17*, 6129-6141; Hewitt, J.M., et al., **1992**, *11*, 1661-1666; Sood, A., et al., *j. Am. Chem. Soc.*, **1990**, *112*, 9000-9001; Vaseur, J.J. et al., *J. Amer. Chem. Soc.*, **1992**, *114*, 4006-4007; Musichi, B., et al., *J. Org. Chem.*, **1990**, *55*, 4231-4233; Reynolds, R.C., et al., *J. Org. Chem.*, **1992**, *57*, 2983-2985; Mertes, M.P., et al., *J. Med. Chem.*, **1969**, *12*, 154-157; Mungall, W.S., et al., *J. Org. Chem.*, **1977**, *42*, 703-706; Stirchak, E.P., et al., *J. Org. Chem.*, **1987**, *52*, 4202-4206; Coull, J.M., et al., *Tet. Lett.*, **1987**, *28*, 745; and Wang, H., et al., *Tet. Lett.*, **1991**, *32*, 7385-7388.

Other modifications can be made to the sugar, to the base, or to the phosphate group of the nucleotide. Representative modifications are disclosed in International Publication Numbers WO 91/10671, published July 25, 1991, WO 92/02258, published February 20, 1992, WO 92/03568, published March 5, 1992, and United States Patents 5,138,045, 5,218,105, 5,223,618 5,359,044, 5,378,825, 5,386,023, 5,457,191, 5,459,255, 5,489,677, 5,506,351, 5,541,307, 5,543,507, 5,571,902, 5,578,718, 5,587,361, 5,587,469, all assigned to the assignee of this application. The disclosures of each of the above referenced publications are herein incorporated by reference.

The attachment of conjugate groups to oligonucleotides and analogs thereof is well documented in the prior art. The compounds of the invention can include

conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, phospholipids, biotin, phenazine, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992, United States Patent No. 5,578,718, issued July 1, 1997, and United States Patent No. 5,218,105. Each of the foregoing is commonly assigned with this application. The entire disclosure of each is incorporated herein by reference.

The properties of many vitamins make them good conjugate groups for inclusion in oligonucleotides, oligonucleosides, or chimeric oligomeric compounds of the invention. α-Tocopherol (vitamin E) and the other tocopherols (beta through zeta) can be conjugated to oligonucleotides to enhance uptake because of their lipophilic character. Also, the lipophilic vitamin, vitamin D, and its ergosterol precursors can be conjugated to oligonucleotides through their hydroxyl groups by first activating the hydroxyls groups to, for example, hemisuccinate esters. Conjugation then is effected to an aminolinker pendant from the oligonucleotide. Other vitamins that can be conjugated to oligonucleotide aminolinkers through hydroxyl groups on the vitamins include thiamine, riboflavin, pyridoxine, pyridoxamine, pyridoxal, deoxypyridoxine. Lipid soluble vitamin K's and related

quinone-containing compounds can be conjugated via carbonyl groups on the quinone ring. The phytol moiety of vitamin K may also serve to enhance binding of the oligonucleotides to cells.

Pyridoxal (vitamin B_6) has specific B_6-binding proteins. The role of these proteins in pyridoxal transport has been studied by Zhang and McCormick, *Proc. Natl. Acad. Sci. USA*, 1991 *88*, 10407. Zhang and McCormick have also shown that a series of N-(4'-pyridoxyl)amines, in which several synthetic amines were conjugated at the 4'-position of pyridoxal, are able to enter cells by a process facilitated by the B6 transporter. They also demonstrated the release of these synthetic amines within the cell. Other pyridoxal family members include pyridoxine, pyridoxamine, pyridoxal phosphate, and pyridoxic acid. Pyridoxic acid, niacin, pantothenic acid, biotin, folic acid and ascorbic acid can be conjugated to oligonucleotides using N-hydroxysuccinimide esters that are reactive with aminolinkers located on the oligonucleotide, as described above for retinoic acid.

Other groups for modifying antisense properties include RNA cleaving complexes, pyrenes, metal chelators, porphyrins, alkylators, hybrid intercalator/ligands and photo-crosslinking agents. RNA cleavers include *o*-phenanthroline/Cu complexes and $Ru(bipyridine)_3^{2+}$ complexes. The $Ru(bpy)_3^{2+}$ complexes interact with nucleic acids and cleave nucleic acids photochemically. Metal chelators are include EDTA, DTPA, and o-phenanthroline. Alkylators include compounds such as iodoacetamide. Porphyrins include porphine, its substituted forms, and metal complexes. Pyrenes include pyrene and other pyrene-based carboxylic acids that could be conjugated using the similar protocols.

Hybrid intercalator/ligands include the photonuclease/intercalator ligand 6-[[[9-[[6-(4-nitro-benzamido)hexyl]amino]acridin-4-yl]carbonyl]amino]hexanoyl-pentafluorophenyl ester. This compound has two noteworthy features: an acridine moiety that is an intercalator and a p-nitro benzamido group that is a photonuclease.

Photo-crosslinking agents include aryl azides such as, for example, N-hydroxysucciniimidyl-4-azidobenzoate (HSAB) and N-succinimidyl-6(-4'-azido-2'-nitrophenyl-amino)hexanoate (SANPAH). Aryl azides conjugated to oligonucleotides effect crosslinking with nucleic acids and proteins upon irradiation, They also crosslink with carrier proteins (such as KLH or BSA), raising antibody against the oligonucleotides.

Vitamins according to the invention generally can be classified as water soluble or lipid soluble. Water soluble vitamins include thiamine, riboflavin, nicotinic acid or niacin, the vitamin B_6 pyridoxal group, pantothenic acid, biotin, folic acid, the B_{12} cobamide coenzymes, inositol, choline and ascorbic acid. Lipid soluble vitamins include the vitamin A family, vitamin D, the vitamin E tocopherol family and vitamin K (and phytols). The vitamin A family, including retinoic acid and retinol, are absorbed and transported to target tissues through their interaction with specific proteins such as cytosol retinol-binding protein type II (CRBP-II), Retinol-binding protein (RBP), and cellular retinol-binding protein (CRBP). These proteins, which have been found in various parts of the human body, have molecular weights of approximately 15 kD. They have specific interactions with compounds of vitamin-A family, especially, retinoic acid and retinol.

In the context of this invention, "hybridization" shall mean hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, also refers to sequence complementarity between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each

other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.* under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

Cleavage of oligonucleotides by nucleolytic enzymes require the formation of an enzyme-substrate complex, or in particular a nuclease-oligonucleotide complex. The nuclease enzymes will generally require specific binding sites located on the oligonucleotides for appropriate attachment. If the oligonucleotide binding sites are removed or blocked, such that nucleases are unable to attach to the oligonucleotides, the oligonucleotides will be nuclease resistant. In the case of restriction endonucleases that cleave sequence-specific palindromic double-stranded DNA, certain binding sites such as the ring nitrogen in the 3- and 7-positions have been identified as required binding sites. Removal of one or more of these sites or sterically blocking approach of the nuclease to these particular positions within the oligonucleotide has provided various levels of resistance to specific nucleases.

This invention provides oligonucleotides possessing superior hybridization properties. Structure-activity relationship studies have revealed that an increase in binding (T_m) of certain 2'-sugar modified oligonucleotides to an RNA

target (complement) correlates with an increased "A" type conformation of the heteroduplex. Furthermore, absolute fidelity of the modified oligonucleotides is maintained. Increased binding of 2'-sugar modified sequence-specific oligonucleotides of the invention provides superior potency and specificity compared to phosphorus-modified oligonucleotides such as methyl phosphonates, phosphate triesters and phosphoramidates as known in the literature.

The only structural difference between DNA and RNA duplexes is a hydrogen atom at the 2'-position of the sugar moiety of a DNA molecule versus a hydroxyl group at the 2'-position of the sugar moiety of an RNA molecule (assuming that the presence or absence of a methyl group in the uracil ring system has no effect). However, gross conformational differences exist between DNA and RNA duplexes.

It is known from X-ray diffraction analysis of nucleic acid fibers (Arnott and Hukins, *Biochem. Biophys. Res. Comm.*, **1970**, 47, 1504) and analysis of crystals of double-stranded nucleic acids that DNA takes a "B" form structure and RNA takes the more rigid "A" form structure. The difference between the sugar puckering (C2' endo for "B" form DNA and C3' endo for "A" form RNA) of the nucleosides of DNA and RNA is the major conformational difference between double-stranded nucleic acids.

The primary contributor to the conformation of the pentofuranosyl moiety is the nature of the substituent at the 2'-position. Thus, the population of the C3'-endo form increases with respect to the C2'-endo form as the electronegativity of the 2'-substituent increases. For example, among 2'-deoxy-2'-haloadenosines, the 2'-fluoro derivative exhibits the largest population (65%) of the C3'-endo form, and the 2'-iodo exhibits the lowest population (7%). Those of adenosine (2'-OH) and deoxyadenosine (2'-H) are 36% and 19%, respectively. Furthermore, the effect of the 2'-fluoro group of adenosine dimers (2'-deoxy-2'-fluoroadenosine - 2'-deoxy-2'-fluoroadenosine) is further correlated to the stabilization of the stacked

conformation. Research indicates that dinucleoside phosphates have a stacked conformation with a geometry similar to that of A-A but with a greater extent of base-base overlapping than A-A. It is assumed that the highly polar nature of the C2'-F bond and the extreme preference for C3'-endo puckering may stabilize the stacked conformation in an "A" structure.

Data from UV hypochromicity, circular dichromism, and ^{1}H NMR also indicate that the degree of stacking decreases as the electronegativity of the halo substituent decreases. Furthermore, steric bulk at the 2'-position of the sugar moiety is better accommodated in an "A" form duplex than a "B" form duplex.

Thus, a 2'-substituent on the 3'-nucleotidyl unit of a dinucleoside monophosphate is thought to exert a number of effects on the stacking conformation: steric repulsion, furanose puckering preference, electrostatic repulsion, hydrophobic attraction, and hydrogen bonding capabilities. These substituent effects are thought to be determined by the molecular size, electronegativity, and hydrophobicity of the substituent.

Studies with a 2'-OMe modification of 2'-deoxy guanosine, cytidine, and uridine dinucleoside phosphates exhibit enhanced stacking effects with respect to the corresponding unmethylated species (2'-OH). In this case, the hydrophobic attractive forces of the methyl group tend to overcome the destablilizing effects of its steric bulk.

Melting temperatures (complementary binding) are increased with the 2'-substituted adenosine diphosphates. It is not clear whether the 3'-endo preference of the conformation or the presence of the substituent is responsible for the increased binding. However, greater overlap of adjacent bases (stacking) can be achieved with the 3'-endo conformation.

While we do not wish to be bound by theory, it is believed that the aminooxyalkyl substituents of the present invention also results in the sugar pucker of the nucleoside being C3'-endo puckering.

Compounds of the invention can be utilized as diagnostics, therapeutics and as research reagents and kits. They can be utilized in pharmaceutical compositions by adding an effective amount of an oligonucleotide of the invention to a suitable pharmaceutically acceptable diluent or carrier. They further can be used for treating organisms having a disease characterized by the undesired production of a protein. The organism can be contacted with an oligonucleotide of the invention having a sequence that is capable of specifically hybridizing with a strand of target nucleic acid that codes for the undesirable protein.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. In general, for therapeutics, a patient in need of such therapy is administered an oligomer in accordance with the invention, commonly in a pharmaceutically acceptable carrier, in doses ranging from 0.01 μg to 100 g per kg of body weight depending on the age of the patient and the severity of the disease state being treated. Further, the treatment may be a single dose or may be a regimen that may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disease state. The dosage of the oligomer may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, or if the disease state has been ablated.

In some cases it may be more effective to treat a patient with an oligomer of the invention in conjunction with other traditional therapeutic modalities. For example, a patient being treated for AIDS may be administered an oligomer in conjunction with AZT, or a patient with atherosclerosis may

be treated with an oligomer of the invention following angioplasty to prevent reocclusion of the treated arteries.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligomers, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to several years.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligomer is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every several years.

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

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

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

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

Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The present invention can be practiced in a variety of organisms ranging from unicellular prokaryotic and eukaryotic organisms to multicellular eukaryotic organisms. Any organism that utilizes DNA-RNA transcription or RNA-protein translation as a fundamental part of its hereditary, metabolic or cellular machinery is susceptible to such therapeutic and/or prophylactic treatment. Seemingly diverse organisms such as bacteria, yeast, protozoa, algae, plant and higher animal forms, including warm-blooded animals, can be treated in this manner. Further, since each of the cells of multicellular eukaryotes also includes both DNA-RNA transcription and RNA-protein translation as an integral part of their cellular activity, such therapeutics and/or diagnostics can also be practiced on such cellular populations. Furthermore, many of the organelles, *e.g.* mitochondria and chloroplasts, of eukaryotic cells also include transcription and translation mechanisms. As such, single cells, cellular populations or organelles also can be included within the definition of organisms that are capable of being treated with the therapeutic or diagnostic oligonucleotides of the invention. As used herein, therapeutics is meant to include both the eradication of a disease state, killing of an organism, e.g. bacterial, protozoan or other infection, or control of aberrant or undesirable cellular growth or expression.

2'-Substituted oligonucleotides were synthesized by standard solid phase nucleic acid synthesis using an automated

synthesizer such as Model 380B (Perkin-Elmer/Applied Biosystems) or MilliGen/Biosearch 7500 or 8800. Triester, phosphoramidite, or hydrogen phosphonate coupling chemistries (*Oligonucleotides: Antisense Inhibitors of Gene Expression*. M. Caruthers, p. 7, J.S. Cohen (Ed.), CRC Press, Boca Raton, Florida, 1989) are used with these synthesizers to provide the desired oligonucleotides. The Beaucage reagent (*J. Amer. Chem. Soc.*, **1990**, *112*, 1253) or elemental sulfur (Beaucage *et al.*, *Tet. Lett.*, **1981**, *22*, 1859) is used with phosphoramidite or hydrogen phosphonate chemistries to provide 2'-substituted phosphorothioate oligonucleotides.

The requisite 2'-substituted nucleosides (A, G, C, T(U), and other modified nucleobases) are prepared, utilizing procedures as described below.

Among other use, the oligonucleotides of the invention are useful in a ras-luciferase fusion system using ras-luciferase transactivation. As described in International Publication Number WO 92/22651, published December 23, 1992 and United States patents 5,582,972 and 5,582,986, commonly assigned with this application, the entire contents of which are herein incorporated by reference, the ras oncogenes are members of a gene family that encode related proteins that are localized to the inner face of the plasma membrane. Ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity. Although the cellular function of ras gene products is unknown, their biochemical properties, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes.

Three ras genes, designated H-ras, K-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras oncogenes have been localized to codons

12, 13, and 61. The most commonly detected activating ras mutation found in human tumors is in codon-12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product. This single amino acid change is thought to abolish normal control of ras protein function, thereby converting a normally regulated cell protein to one that is continuously active. It is believed that such deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth.

In addition to modulation of the ras gene, the oligonucleotides of the present invention that are specifically hybridizable with other nucleic acids can be used to modulate the expression of such other nucleic acids. Examples include the raf gene, a naturally present cellular gene which occasionally converts to an activated form that has been implicated in abnormal cell proliferation and tumor formation. Other examples include those relating to protein kinase C (PKC) that have been found to modulate the expression of PKC, those related to cell adhesion molecules such as ICAM, those related to multi-drug resistance associated protein, and viral genomic nucleic acids include HIV, herpes viruses, Epstein-Barr virus, cytomegalovirus, papillomavirus, hepatitis C virus and influenza virus (see United States patents 5,166,195, 5,242,906, 5,248,670, 5,442,049, 5,457,189, 5,510,476, 5,510,239, 5,514,577, 5,514,786, 5,514,788, 5,523,389, 5,530,389, 5,563,255, 5,576,302, 5,576,902, 5,576,208, 5,580,767, 5,582,972, 5,582,986, 5,591,720, 5,591,600 and 5,591,623, commonly assigned with this application, the disclosures of which are herein incorporated by reference).

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

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The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

General

All reagents and solvents were purchased from Aldrich Chemicals unless otherwise noted. NMR spectra were obtained

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with the following instruments: ^{1}H-NMR: Varian Gemini-200 (199.975 MHZ) or Varian Unity 400 (399.952 MHZ). ^{13}C-NMR: Varian Gemini-200 (50.289 MHZ). ^{31}P-NMR: Varian Gemini-200 (79.990 MHZ) or Varian Unity 400 (159.981 MHZ). NMR spectra were recorded using either deuteriochloroform or dimethylsulfoxide-d_6 as solvent (tetramethylsilane or phosphoric acid internal standard). The following abbreviations were used to designate the multiplicity of individual signals: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, br s = broad singlet. Mass spectra were acquired on a VG 70-SEQ instrument (VG Analytical (Fisons)), using fast atom bombardment ionization (7 kV Xe atoms). Solvent ratios for column chromatography are given as volume/volume. Evaporations of solvents were performed in vacuo (60 torr) at 35 °C unless otherwise specified.

EXAMPLE 1

Methyl-2-O-(2-ethylacetyl)-3,5-bis-O-(2,4-dichlorobenzyl)-α-D-ribofuranoside (3, Figure 1)

Compound 2 (Figure 1) (multigram quantities of 2 were prepared from 1 via the literature procedure, Martin, P. *Helv. Chem.Acta*, 1995, *78*, 486-504) was dissolved in DMF (86 mL) with cooling to 5 °C, and NaH (60% dispersion, 1.38 g, 34.38 mmol) was added. The reaction mixture was stirred at 5 °C for 5 minutes then warmed to ambient temperature and stirred for 20 minutes after which time the reaction mixture was cooled to 5 °C and ethylbromoacetate (3.81 mL, 34.4 mmol) was added dropwise resulting in the evolution of gas. The reaction mixture was allowed to warm to ambient temperature and stirred for 3 hours after which time the mixture was cooled to 5 °C and the pH was adjusted to 3 with saturated aqueous NH_4Cl. The solvent was evaporated in vacuo to give a syrup which was dissolved in EtOAc (200 mL), washed with water and then brine. The organic layer was separated, dried with $MgSO_4$, and the solvent was evaporated in vacuo to give an oil. The oil was purified by flash chromatography using hexanes-EtOAc, 60:40, to give the title compound (3) as an oil (15.52 g, 95%). ^{1}H

NMR ($CDCl_3$): δ 7.58-7.18 (m, 6H), 5.05 (d, J = 3.8 Hz, 1H), 4.79 (q, J_{AB} = 13.7 Hz, 2H), 4.57 (d, J = 2.8 Hz, 2H), 4.31-4.16 (m, 5H), 4.03 (m, 2H), 3.62 (d, 2H), 3.50 (s, 3H), 1.28 (t, 3H). ^{13}C NMR ($CDCl_3$): δ 170.0, 134.2, 133.6, 133.5, 130.3, 129.8, 129.1, 128.8, 127.1, 102.1, 81.4, 78.9, 76.6, 70.6, 70.0, 69.3, 67.6, 61.0, 55.6, 14.2. Anal. Calcd for $C_{24}H_{26}Cl_4O_7 \cdot H_2O$: C, 49.17; H, 4.81. Found: C, 49.33; H, 4.31.

EXAMPLE 2

1-[2'-O-(2-ethylacetyl)-3',5'-bis-O-(2,4-dichlorobenzyl)-β-D-ribofuranosyl]thymine (4, Figure 1)

Thymine (6.90 g, 54.6 mmol) was suspended in anhydrous dichloroethane (136 mL) and bis-trimethylsilylacetamide (40.5 mL, 164 mmol) was added. The reaction mixture was heated to reflux temperature for 10 minutes to give dissolution. After cooling to ambient temperature, the solution was added to compound 3 with stirring. Trimethylsilyl trifluoromethanesulfonate (6.86 mL, 35.5 mmol) was added and the reaction mixture was heated to reflux for 6 hours. The mixture was cooled to 5 °C and the pH was adjusted to 7 by the slow addition of saturated $NaHCO_3$. The mixture was extracted with CH_2Cl_2 (3 x 150 mL) and the organic extracts were combined, washed with brine, and the solvent was evaporated in vacuo to give an oil. The oil was dissolved in CH_2Cl_2 and purified by flash chromatography using hexanes-EtOAc, 45:55, to provide the title compound (4) as an oil (7.92 g, 44%). (The α-anomer was contained in a later fraction). ^1H NMR (400 MHZ, $CDCl_3$): δ 8.25 (s, 1H), 7.67 (s, 1H), 7.46-7.21 (m, 6H), 5.94 (d, J = 1.6 Hz, 1H), 4.80 (q, J_{AB} = 12.4 Hz, 2H), 4.70-4.18 (m, 9H), 4.02 (d, 1H), 3.75 (d, 1H), 1.58 (s, 3H), 1.26 (t, 3H). ^{13}C NMR ($CDCl_3$): δ 170.1, 164.3, 150.3, 135.5, 134.5, 134.2, 134.1, 133.8, 133.5, 130.7, 130.2, 129.4, 129.0, 127.1, 110.3, 88.4, 80.8, 80.5, 74.7, 70.1, 68.9, 68.0, 66.2, 60.9, 14.1, 12.1. Anal. Calcd for $C_{28}H_{28}Cl_4N_2O_8 \cdot H_2O$: C, 49.43; H, 4.44; N, 4.12. Found: C, 49.25; H, 4.10; N, 3.94.

EXAMPLE 3

1-[2'-O-(2-hydroxyethyl)-3',5'-bis-O-(2,4-dichlorobenzyl)-β-D-ribofuranosyl]thymine (5, Figure 1)

Compound **4** (9.92 g, 15.0 mmol) was dissolved in hot EtOH (150 mL) and the solution was cooled to ambient temperature in a water bath. To the solution was cautiously added $NaBH_4$ (1.13 g, 30.0 mmol) over 10 minutes. After 3 hours additional $NaBH_4$ (282 mg, 7.45 mmol) was added the mixture was stirred for 1 hour and left to stand for 8 hours. The pH was adjusted to 4 by addition of Saturated NH_4Cl (25 mL) to give a gum. The solvent was decanted and evaporated in vacuo to afford a white solid which was dissolved in CH_2Cl_2 (250 mL). The gum was dissolved with saturated aqueous $NaHCO_3$ and this solution was gently extracted with the CH_2Cl_2 containing the product. The organic layer was separated and the aqueous layer was extracted again with CH_2Cl_2 (2 x 50 mL). After combining the organic layers, the solvent was dried over $MgSO_4$ and evaporated in vacuo to afford a white foam. The foam was dissolved in CH_2Cl_2 and purified by flash chromatography using hexanes-EtOAc, 20:80, to give the title compound (5) as a white foam (8.39 g, 90%). ^1H NMR ($CDCl_3$): δ 10.18 (s, 1H), 7.66 (s, 1H), 7.39-7.20 (m, 6H), 5.96 (s, 1H), 4.76-3.62 (m, 14H), 1.58 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 164.0, 150.8, 135.2, 134.6, 134.2, 134.1, 133.5, 133.4, 130.2, 129.4, 129.0, 127.1, 110.6, 88.6, 81.0, 80.7, 75.2, 72.0, 70.1, 68.9, 68.1, 61.9, 12.1.

EXAMPLE 4

1-[2'-O-(2-phthalimido-N-hydroxyethyl)-3',5'-bis-O-(2,4-dichlorobenzyl)-β-D-ribofuranosyl]thymine (6, Figure 1)

Compound **5** was dried by coevaporation with anhydrous acetonitrile followed by further drying in vacuo (0.1 torr) at ambient temperature for 12 h. The dried material (8.39 g, 13.53 mmol) was dissolved in freshly distilled THF (97 mL), PPh_3 (3.90 g, 14.9 mmol), and N-hydroxyphthalimide (2.43 g, 14.9 mmol) was added. The reaction mixture was cooled to -78 °C, and diethyl azodicarboxylate (2.34 mL, 14.9 mmol) was added. The reaction mixture was warmed to ambient temperature

and the solvent was evaporated in vacuo to give a foam. The foam was dissolved in EtOAc (100 mL) and washed with saturated aqueous $NaHCO_3$ (3 x 30 mL). The organic layer was separated, washed with brine, dried over $MgSO_4$, and the solvent evaporated to give a foam. The foam was purified by flash chromatography using CH_2Cl_2-acetone, 85:15, to give the title compound (6) as a white foam (3.22 g, 31%). A second chromatographic purification provided additional 6 as a white foam (5.18 g, 50%). ^1H NMR (400 MHZ, $CDCl_3$): δ 9.0 (s, 1H), 7.8 (m, 11H), 5.95 (s, 1H), 4.84-3.70 (m, 13H), 1.60 (s, 3H). ^{13}C NMR (100 MHZ, $CDCl_3$): δ 163.7, 163.5, 150.2, 138.0, 135.6, 134.5, 134.1, 134.0, 133.9, 133.7, 133.6, 130.6. 130.4, 130.1, 129.8, 129.4, 129.1, 129.0, 128.8, 127.2, 123.5, 110.4, 88.2, 81.0, 80.9, 77.6, 75.4, 70.2, 68.9, 68.4, 68.1., 12.1. LRMS (FAB+) *m/z* : 766 (M + H). LRMS (FAB-) *m/z* : 764 (M - H).

EXAMPLE 5

1-[2'-O-(2-phthalimido-N-oxyethyl)-3',5'-bis-O-(2,4-dichlorobenzyl)-β-D-ribofuranosyl]thymine (7, Figure 2)

Compound 6 (1.79 g, 2.34 mmol) was dissolved in CH_2Cl_2 (12 mL), the solution was cooled to -78 °C and 1.0 M boron trichloride (5.15 mL, 5.15 mmol) in CH_2Cl_2 was added and the reaction mixture was kept at 5 °C for 1.5 hours. Additional 1.0 M boron trichloride (5.15 mL, 5.15 mmol) in CH_2Cl_2 was added and the solution was stirred at 5° for an additional 1.5 hours. The pH was adjusted to 7 with saturated aqueous $NaHCO_3$ (30 mL). After dilution with CH_2Cl_2 (100 mL), the organic layer was separated, and the aqueous layer was extracted with $CHCl_3$ (5 x 25 mL) and then EtOAc (3 x 25 mL). The organic layers were combined, dried over Na_2SO_4, and evaporated in vacuo to give an oil. The oil was purified by flash chromatography using CH_2Cl_2-acetone, 45:55, to provide the title compound (7) as a white foam (619 mg, 59%). ^1H NMR ($CDCl_3$): δ 8.8 (br, 1H), 7.88-7.75 (m, 4H), 7.50 (s, 1H), 5.70 (d, J = 4 Hz, 1H), 4.45-3.75 (m, 11H), 2.95 (br, 1H), 1.90 (s, 3H). ^{13}C NMR (100 MHZ, $CDCl_3$): δ 164.3, 163.7, 150.6, 137.4, 134.7, 128.5, 123.6, 110.5, 89.7, 84.7, 81.9, 77.6, 68.5, 68.4,

61.0, 12.3. LRMS (FAB+) *m/z* : 448 (M + H). LRMS (FAB-) *m/z* : 446 (M - H).

EXAMPLE 6

1-[2'-O-(2-phthalimido-N-oxyethyl)-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]thymine (8, Figure 2)

Compound 7 was dried by coevaporation with anhydrous acetonitrile followed by further drying in vacuo (0.1 torr) at ambient temperature for 12 hours. The dried material (619 mg, 1.38 mmol) was dissolved in anhydrous pyridine (7 mL) and 4,4'-dimethoxytrityl chloride (514 mg, 1.52 mmol) was added. After 2 hours additional 4,4'-dimethoxytrityl chloride (257 mg, 0.76 mmol) was added. The solution was stirred for 2 hours and a final addition of 4,4'-dimethoxytrityl chloride (257 mg, 0.76 mmol) was made. After 12 h MeOH (10 mL) was added to the reaction mixture, it was stirred for 10 min and the solvent was evaporated in vacuo to give an oil which was coevaporated with toluene. The oil was purified by flash chromatography by pre-treating the silica with CH_2Cl_2-acetone-pyridine, 80:20:1, then using CH_2Cl_2-acetone, 80:20 to afford the title compound (**8**) as a yellow solid (704 mg, 68%). ^1H NMR ($CDCl_3$): δ 7.8-6.8 (m, 18H), 5.94 (d, J = 2.2 Hz, 1H), 4.57-4.12 (m, 7H), 3.78 (s, 6H), 3.53 (m, 2H), 1.34 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 164.3, 163.8, 158.6, 150.6, 144.4, 135.5, 135.4, 134.7, 130.1, 128.7, 128.2, 128.0, 127.1, 123.7, 113.3, 110.9, 87.9, 86.7, 83.2, 68.7, 68.5, 61.7, 55.2, 11.9. LRMS (FAB+) *m/z* : 750 (M + H). LRMS (FAB-) *m/z* : 748 (M - H). Anal. Calcd for $C_{41}H_{39}N_3O_{11}\cdot H_2O$: C, 65.14; H, 5.38; N, 5.47. Found: C, 63.85; H, 5.16; N, 5.14. Anal. Calcd for $C_{41}H_{39}N_3O_{11}$: C, 65.68; H, 5.24; N, 5.60. Found: C, 65.23; H, 5.27; N, 5.45.

EXAMPLE 7

1-[2'-O-(2-phthalimido-N-oxyethyl)-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]thymine-3'-[(2-cyanoethyl)-N,N-diisopropyl-phosphoramidite] (9, Figure 2)

Compound 8 was dried by coevaporation with anhydrous pyridine (2 x 20 mL), then further dried in vacuo (0.1 torr)

at ambient temperature for 12 hours. The dried material (704 mg, 0.939 mmol) was dissolved in CH_2Cl_2 (9 mL), diisopropylamine tetrazolide (80.4 mg, 0.47 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.33 mL, 1.03 mmol) with stirring. After 2 hours at ambient temperature additional 2-cyanoethyl-N,N,N',N'-tetra-isopropylphosphorodiamidite (0.33 mL, 1.03 mmol) was added and the solution was stirred for 20 hours. The solvent was evaporated in vacuo to give an oil which was purified by flash chromatography by pre-treating the silica with CH_2Cl_2-acetone-pyridine, 85:15:1, then using CH_2Cl_2-acetone, 85:15 to afford the title compound (9) as an oil (704 mg, 68%). The product was coevaporated with anhydrous acetonitrile (2 x 30 mL) and CH_2Cl_2 (2 x 30 mL) to afford a yellow foam. ^1H NMR ($CDCl_3$): δ 8.6 (br, 1H), 7.78-6.82 (m, 18H), 6.06 (m, 1H), 4.6-3.3 (m, 14H), 3.75 (s, 6H), 2.66 (m, 1H), 2.37 (m, 1H), 1.36 (s, 3H), 1.16 (m, 12H). ^{31}P NMR ($CDCl_3$): δ 150.5, 151.2. LRMS (FAB+) *m/z* : 950 (M + H). LRMS (FAB-) *m/z* : 948 (M - H). Anal. Calcd for $C_{50}H_{56}N_5O_{12}P \cdot H_2O$: C, 62.04; H, 6.04; N, 7.24. Found: C, 62.20; H, 5.94; N, 7.34.

EXAMPLE 8

2'-O-(2-ethylacetyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (11, Figure 3)

Adenosine (30.00 g, 112 mmol) was dissolved in hot anhydrous DMF (600 mL) and the solution was cooled to ambient temperature. NaH (60% dispersion oil, 4.94 g, 124 mmol) was added and the mixture was stirred with a mechanical stirrer for 1 hour. The resulting suspension was cooled to 5 °C and ethylbromoacetate (13.7 mL, 124 mmol) was added. The resulting solution was stirred for 12 hours at ambient temperature and the solvent was evaporated in vacuo to give a residue which contained 2'-O-(2-ethylacetyl)adenosine (10) and the putative 3'-O-isomer. This material was coevaporated with pyridine to give a foam which was dissolved in anhydrous pyridine (400 mL). 1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (39.52 mL, 124 mmol) was added and the solution was stirred for 24 hours at

ambient temperature. The solvent was evaporated in vacuo to give an oil which was dissolved in EtOAc (500 mL) and washed with brine three times. The organic layer was separated, dried over $MgSO_4$, and the solvent was evaporated in vacuo to afford an oil. The oil was purified by flash chromatography using hexanes-EtOAc, 80:20, to give the title compound (11) as an oil (14.63 g, 22%). ^1H NMR ($CDCl_3$): δ 8.26 (s, 1H), 8.07 (s, 1H), 6.20 (br s, 2H), 4.91 (dd, $J_{1',2'}$ = 4.7 Hz, $J_{2',3'}$ = 9.3 Hz, 1H), 4.64-3.97 (m, 8H), 1.22 (t, 3H), 1.05 (m, 28 H). ^{13}C NMR ($CDCl_3$): δ 170.0, 155.5, 152.8, 149.0 139.3, 120.2, 88.6, 82.2, 81.1, 69.9, 68.3, 60.8, 60.0, 17.2, 14.0, 12.7. Anal. Calcd for $C_{26}H_{45}N_5O_7Si_2$: C, 52.41; H, 7.61; N, 11.75, Si, 9.43. Found: C, 52.23; H, 7.34; N, 11.69.

EXAMPLE 9

2'-O-(2-hydroxyethyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (12, Figure 3)

Compound 11 (4.175 g, 7.01 mmol) was dissolved in ethanol (95%, 40 mL) and the resulting solution was cooled to 5 °C. $NaBH_4$ (60% oil dispersion, 0.64 g, 16.8 mmol) was added, and the mixture was allowed to warm to ambient temperature. After stirring for 12 hours CH_2Cl_2 (200 mL) was added and the solution was washed with brine twice and the organic layer was separated. The organic layer was dried over $MgSO_4$, and the solvent was evaporated in vacuo to give an oil. The oil was purified by flash chromatography using EtOAc-MeOH, 95:5, to afford the title compound (12) as an oil (0.368 g, 9.5%). ^1H NMR ($CDCl_3$): δ 8.31 (s, 1H), 8.14 (s, 1H), 6.18 (br s, 2H), 6.07 (s, 1H), 4.62 (dd, $J_{1',2'}$ = 4.6 Hz, $J_{2',3'}$ = 9.4 Hz, 1H), 4.3-3.5 (m, 8H), 1.03 (m, 28H). ^{13}C NMR ($CDCl_3$): δ 155.5, 153.0, 148.7, 138.3, 120.3, 89.2, 82.7, 81.4, 73.5, 69.3, 61.8, 59.7, 17.2, 17.0, 16.8, 13.4, 12.9, 12.8, 12.6. LRMS (FAB+) *m/z* : 554 (M + H), 686 (M + Cs+).

EXAMPLE 10

2'-O-(2-Phthalimido-N-hydroxyethyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (13, Figure 4)

To a solution of compound 12 (0.330 g, 0.596 mmol) in anhydrous THF (10 mL) was added triphenylphosphine (0.180 g, 0.685 mmol) and N-hydroxyphthalimide (0.112 g, 0.685 mmol). To this mixture diethyl azodicarboxylate (0.11 mL, 685 mmol) was added dropwise at 5 °C. After stirring for 3 hours at ambient temperature, the solvent was evaporated to give an oil. The oil was dissolved in EtOAc and washed with saturated aqueous $NaHCO_3$ (x3) and brine. The organic layer was separated, dried over $MgSO_4$. The solvent was evaporated in vacuo to give an oil. The oil was purified by flash chromatography using EtOAc-MeOH, 95:5, to give the title compound (13) as an oil (0.285 g, 68%). ^1H NMR ($CDCl_3$): δ 8.21 (s, 1H), 8.05 (s, 1H), 7.8-7.45 (m, 4H), 6.00 (s, 1H), 5.88 (br s, 2H), 4.92 (dd, $J_{1',2'}$ = 4.6, $J_{2',3'}$ = 9.0 Hz), 4.5-3.9 (m, 8H), 1.0 (m, 28H). ^{13}C NMR ($CDCl_3$): δ 163, 155.3, 152.8, 149, 139.6, 134.3, 123.4, 120, 88.7, 82.7, 81.1, 77.4, 70.2, 69.5, 60.1, 17.4, 17.2, 17.0, 16.9, 13.3, 12.9, 12.7, 12.6. LRMS (FAB+) *m/z* : 699 (M + H).

EXAMPLE 11

N6-Benzoyl-2'-O-(2-phthalimido-N-hydroxyethyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (14, Figure 4)

To a solution of compound 13 (1.09 g, 1.97 mmol) in anhydrous pyridine (19 mL) cooled to 5 °C was added benzoyl chloride (1.14 mL, 9.8 mmol) and the resulting mixture was stirred at ambient temperature for 12 hours. After cooling the mixture to 5 °C, cold water (3.8 mL) was added, the mixture was stirred for 15 minutes, and conc NH_4OH (3.8 mL) was added. After stirring for 30 minutes at 5 °C the solvent was evaporated to give a residue which was dissolved in water and extracted with CH_2Cl_2 three times. The organic extracts were combined, dried over $MgSO_4$, and evaporated in vacuo to afford an oil. The oil was purified by flash chromatography using

hexanes-EtOAc, 50:50, then 20:80, to give the title compound (14) as an oil (0.618 g, 48%). ^{1}H NMR ($CDCl_3$): δ 9.2 (br s, 1H), 8.69 (s, 1H), 8.27 (s, 1H), 8.0-7.4 (m, 9H), 6.12 (s, 1H), 4.95 (dd, $J_{1',2'}$ = 4.7 Hz, $J_{2',3'}$ = 9.1 Hz, 1H), 4.5-4.0 (m, 8H), 1.06 (m, 28H). ^{13}C NMR ($CDCl_3$): δ 164.4, 163.3, 152.5, 150.8, 149.3, 142.1, 134.4, 133.7, 132.6, 132.1, 128.7, 128.2, 127.7, 123.4, 88.9, 82.7, 81.3, 77.5, 70.1, 69.6, 60.0, 17.2, 17.0, 16.8, 13.3, 12.8, 12.7, 12.6. LRMS (FAB+) *m/z* : 803 (M + H).

EXAMPLE 12

N^6-Benzoyl-2'-O-(2-phthalimido-N-hydroxyethyl)adenosine (15, Figure 4)

To a solution of compound 14 (0.680 g, 0.847 mmol) in THF (20 mL) in a polyethylene reaction vessel at 5 °C was added HF-pyridine (70%, 0.48 mL, 16.9 mmol) and the resulting mixture was warmed to ambient temperature. After stirring for 12 hours the solvent was evaporated in vacuo, EtOAc was added, the solution was washed with water, and the aqueous layer was separated and extracted with EtOAc. The organic layers were combined, dried over $MgSO_4$, and the solvent was evaporated in vacuo to give the title compound (15) as a solid (408 mg, 86%). ^{1}H NMR (DMSO-d_6): δ 11.2 (br s, 1H), 8.71 (s, 1H), 8.67 (s, 1H), 8.0-7.5 (m, 9H), 6.11 (d, J 1',2' = 5.7 Hz), 5.23 (d, 1H), 5.14 (t, 1H), 4.66 (t, 1H), 4.35 (m, 3H), 3.90 (m, 3H), 3.6 (m, 2H). ^{13}C NMR (DMSO-d_6): δ 163.5, 152.0, 143.2, 135.0, 132.6, 131.9, 131.7, 129.3, 128.7, 128.5, 123.4, 86.3, 85.8, 81.3, 76.8, 69.0, 68.7, 61.3. LRMS (FAB+) *m/z* : 561 (M + H, 583 (M + Na+).

EXAMPLE 13

N^6-Benzoyl-2'-O-(2-phthalimido-N-oxyethyl)-5'-O-(4,4'-dimethoxytrityl)adenosine (16, Figure 4)

To a solution of compound 15 (0.258 g, 0.46 mmol) in anhydrous pyridine (5 mL) was added 4,4'-dimethoxytrityl chloride (0.179 g, 0.53 mmol) and the solution was stirred for 12 hours at ambient temperature. Water was added and the mixture was extracted with EtOAc three times. The organic

extracts were combined, evaporated in vacuo, and dried over $MgSO_4$. The resulting oil was purified by flash chromatography using hexanes-EtOAc, 90:10, to give the title compound (16) as an oil (0.249 g, 63%). ^1H NMR ($CDCl_3$): δ 9.16 (br s, 1H), 8.68 (s, 1H), 8.28 (s, 1H), 8.1-6.8 (m, 22H), 6.26 (d, J 1',2' = 4.0 Hz, 1H), 4.76 (m, 1H), 4.60 (m, 1H), 4.4-4.3 (m, 3H), 4.13-4.0 (m, 3H), 3.77 (s, 6H), 3.48 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 164.5, 163.6, 158.5, 152.6, 151.4, 149.5, 144.5, 141.9, 135.7, 134.7, 132.7. 130.1, 128.8, 128.2, 127.8, 126.9, 123.7, 113.2, 87.2, 84.1, 82.6, 69.9, 69.0, 63.0, 60.3, 55.2. HRMS (FAB+) *m/z* (M + Cs+) calcd for $C_{48}H_{42}N_5O_{10}$ 995.2017, found 995.2053 (M + Cs+).

EXAMPLE 14

N^6-Benzoyl-2'-O-(2-phthalimido-N-oxyethyl)-5'-O-(4,4'-dimethoxytrityl)adenosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (17, Figure 4)

To a solution of compound 16 (0.300 g, 0.348 mmol) in CH_2Cl_2 (10 mL) was added diisopropylamine tetrazolide (0.030 g, 0.174 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropyl-phosphorodiamidite (0.13 mL, 0.418 mmol). After stirring for 12 hours at ambient temperature additional diisopropylamine tetrazolide (0.060 g, 0.348 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.26 mL, 0.832 mmol) were added in two portions over 24 hours. After 24 hours CH_2Cl_2-NEt_3, 100:1, was added and the mixture was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was separated, dried over $MgSO_4$, and the solvent was evaporated in vacuo. The resulting oil was purified by flash chromatography by pre-treating the silica with hexanes-EtOAc-NEt_3, (40:60:1), then using the same solvent system to give the title compound (17) as an oil (203 g, 55%). ^1H NMR ($CDCl_3$): δ 6.27 (m, 1H). ^{31}P NMR ($CDCl_3$): δ 151.0, 150.5. HRMS (FAB+) *m/z* (M + Cs+) calcd for $C_{57}H_{59}N_8O_{11}P$ 1195.3095, found 1195.3046 (M + Cs+).

EXAMPLE 15

2'-O-(2-aminooxyethyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (18, Figure 5)

To a solution of compound 13 (0.228 g, 0.326 mmol) in CH_2Cl_2 (5 mL) at 5 °C was added methylhydrazine (0.017 mL, 0.326 mmol) with stirring for 2 hours. The mixture was filtered to remove a precipitate and the filtrate was washed with water and brine. The organic layer was separated, dried over $MgSO_4$, and the evaporated in vacuo to give the title compound (18) as an oil (186 mg). The oil was of sufficient purity for subsequent reactions. ^1H NMR ($CDCl_3$): δ 8.31 (s, 1H), 8.15 (s, 1H), 6.07 (s, 1H), 5.78 (br s, 2H), 4.70 (dd, J 1',2' = 4.4 Hz, J 2',3' = 9.0 Hz, 1H), 4.3-3.9 (m, 8H), 1.9 (br, 2H), 1.0 (m, 28H). LRMS (FAB+) *m/z* : 569 (M + H), 702 (M + Cs^+).

EXAMPLE 16

2'-O-(2-O-Formaldoximylethyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (19, Figure 5)

To a solution of compound 18 (0.186 g, 0.326 mmol) in EtOAc (2 mL) and MeOH (2 mL) was added formaldehyde (aqueous 37%, 0.028 mL, 0.342 mmol) with stirring at ambient temperature for 3 hours. The solvent was evaporated in vacuo to give the title compound (19) as an oil (189 mg). The oil was of sufficient purity for subsequent reactions. ^1H NMR ($CDCl_3$): δ 8.31 (s, 1H), 8.09 (s, 1H), 6.97 (d, J = 8.3 Hz, 1H), 6.38 (d, J = 8.3 Hz, 1H), 6.01 (s, 1H), 5.66 (br s, 2H), 4.77 (dd, $J_{1',2'}$ = 4.7 Hz, $J_{2',3'}$ = 9.3 Hz), 4.3-4.0 (m, 8H), 1.0 (m, 28H). LRMS (FAB+) *m/z* : 581 (M + H), 713 (M + Cs^+).

EXAMPLE 17

N^6-Benzoyl-2'-O-(2-O-formaldoximylethyl)-3',5'-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)adenosine (20, Figure 5)

To a solution of compound 19 (0.189 g, 0.326 mmol) in pyridine (5 mL) at 5 °C was added benzoyl chloride (0.19 mL, 1.63 mmol) and the resulting solution was stirred at ambient temperature for 3 hours. The solution was cooled to 5 °C and

concentrated NH_4OH (1.5 mL) was added with stirring for 1 hour. The solvent was evaporated in vacuo to give an oil which was dissolved in CH_2Cl_2. The solution was washed with water and the organic layer was separated, dried with $MgSO_4$, and the solvent was evaporated to give the title compound (20) (223 mg) as an oil which was of sufficient purity for subsequent reactions. ^1H NMR ($CDCl_3$): δ 9.30 (br, 1H), 8.79 (s, 1H), 8.31 (s, 1H), 8.1-7.2 (m, 5H), 7.00 (d, 1H), 6.39 (d, 1H), 6.09 (s, 1H), 4.77 (dd, 1H), 4.4-3.9 (m, 8H), 1.1 (m, 28H).

EXAMPLE 18

N^6-Benzoyl-2'-O-(2-O-formaldoximylethyl)adenosine (21, Figure 5)

To a solution of compound 20 (223 mg, 0.326 mmol) in THF (10 mL) in a polyethylene reaction vessel at 5 °C was added HF-pyridine (70%, 0.19 mL, 6.5 mmol) and the mixture was allowed to warm to ambient temperature. After stirring for 48 hours the solvents were evaporated in vacuo to give a residue which was dissolved in EtOAc and washed with water. The organic layer was separated, the aqueous layer was extracted with EtOAc, and the organic layers were combined, dried over $MgSO_4$, and evaporated in vacuo. The resulting residue was purified by flash chromatography using EtOAc-MeOH, 95:5, to give the title compound (21) as a solid (24 mg, 17% from 13). ^1H NMR ($CDCl_3$): δ 9.05 (br s, 1H), 8.77 (s, 1H), 8.13 (s, 1H), 7.9-7.2 (m), 6.26 (d, J = 10.7 Hz, 1H), 6.03 (d, $J_{1',2'}$ = 7.8 Hz), 4.88 (dd, J = 4.6 Hz, J = 7.9 Hz, 1H), 4.6-3.7 (m, 10H). LRMS (FAB+) *m/z* : 443 (M + H). LRMS (FAB-) *m/z* : 441 (M - H).

EXAMPLE 19

N6-Benzoyl-2'-O-(2-O-formaldoximylethyl)-5'-O-(4,4'-dimethoxytrityl)adenosine (21A, Figure 5)

To a solution of compound 21 (0.34 g, 0.768 mmol) in pyridine (7 mL) was added 4,4'-dimethoxytrityl chloride (0.312 g, 0.922 mmol) and the reaction mixture was stirred at ambient temperature for 5 hours. Additional amounts of 4,4'-dimethoxy-

trityl chloride (520 mg, 1.54 mmol and 340 mg, 0.768 mmol) were added over 24 hours. The solvent was evaporated, the crude product was dissolved in EtOAc, and washed with water. The organic layer was separated, dried over $MgSO_4$ and the solvent was evaporated in vacuo. The crude material was purified by column chromatography using EtOAc-Hexanes-NEt_3, 80:20:0.5, v/v/v, followed by, EtOAc-NEt3, 100:0.5, v/v, as solvent to give the title compound (21A) as an oil (0.269 g, 47%). ^1H NMR ($CDCl_3$): δ 8.99 (br s, 1H), 8.74 (s, 1H), 8.1-6.8 (m, 18H), 7.00 (d, 1H), 6.43 (d, 1H), 6.19 (d, 1H), 4.72 (m, 1H), 4.48 (m, 1H), 4.23 (m, 3H), 4.1 (m, 1H), 3.9 (m, 1H), 3.78 (s, 6H), 3.45 (m, 2H), 3.15 (d, 1H). HRMS (FAB+) *m/z* (M + Cs+) calcd for $C_{41}H_{40}N_6O_8$ 877.1962, found 877.1988 (M + Cs+).

EXAMPLE 20

2'-O-Allyl-5'-O-dimethoxytrityl-5-methyluridine

In a 100 mL stainless steel pressure reactor, allyl alcohol (20 mL) was slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring. Hydrogen gas rapidly evolved. Once the rate of bubbling subsided, 2,2'-anhydro-5-methyluridine (1.0 g, 0.4.2 mmol) and sodium bicarbonate (6 mg) were added and the reactor was sealed. The reactor was placed in an oil bath and heated to 170 °C internal temperature for 18 hours. The reactor was cooled to room temperature and opened. Tlc revealed that all the starting material was gone (starting material and product Rf 0.25 and 0.60 respectively in 4:1 ethyl acetate/methanol on silica gel). The crude solution was concentrated, coevaporated with methanol (50 mL), boiling water (15 mL), absolute ethanol (2x25 mL) and then the residue was dried to 1.4 g of tan foam (1 mm Hg, 25 °C, 2 hours). A portion of the crude nucleoside (1.2 g) was used for the next reaction step without further purification. The residue was coevaporated with pyridine (30 mL) and redissolved in pyridine (30 mL). Dimethoxytrityl chloride (1.7 g, 5.0 mmol) was added in one portion at room temperature. After 2 hours the reaction was quenched with methanol (5 mL), concentrated in vacuo and partitioned between a solution of

saturated sodium bicarbonate and ethyl acetate (150 mL each). The organic phase was separated, concentrated and the residue was subjected to column chromatography (45 g silica gel) using a solvent gradient of hexanes-ethyl acetate-triethylamine (50:49:1) to (60:39:1). The product containing fractions were combined, concentrated, coevaporated with acetonitrile (30 mL) and dried (1 mm hg , 25 °C, 24 hours) to 840 mg (34% two-step yield) of white foam solid. The NMR was consistent with the unmethylated uridine analog reported in the literature.

EXAMPLE 21

2'-O-(2-hydroxyethyl)-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Allyl-5'-O-dimethoxytrityl-5-methyluridine (1.0 g, 1.6 mmol), aqueous osmium tetroxide (0.15 M, 0.36 mL, 0.0056 mmol, 0.035 eq) and 4-methylmorpholine N-oxide (0.41 g, 3.5 mmol, 2.15 eq) were dissolved in dioxane (20 mL) and stirred at 25 °C for 4 hours. Tlc indicated complete and clean reaction to the diol (Rf of starting to diol 0.40 to 0.15 in dichloromethane/methanol 97:3 on silica). Potassium periodate (0.81 g, 3.56 mmol, 2.2 eq) was dissolved in water (10 mL) and added to the reaction. After 17 hours the tlc indicated a 90% complete reaction (aldehyde Rf 0.35 in system noted above). The reaction solution was filtered, quenched with 5% aqueous sodium bisulfite (200 mL) and the product aldehyde was extracted with ethyl acetate (2x200 mL). The organic layers were combined, washed with brine (2x100 mL) and concentrated to an oil. The oil was dissolved in absolute ethanol (15 mL) and sodium borohydride (1 g) was added. After 2 hours at 25 °C the tlc indicated a complete reaction. Water (5 mL) was added to destroy the borohydride. After 2 hours the reaction was stripped and the residue was partitioned between ethyl acetate and saturated sodium bicarbonate solution (50 mL each). The organic layer was concentrated in vacuo and the residue was columned (silica gel 30 g, dichloromethane-methanol 97:3). The product containing fractions were combined and stripped and dried to 0.50 g (50 %) of white foam. The NMR was consistent with that of material prepared by the glycosylation route.

EXAMPLE 22

2'-O-(2-hydroxyethyl)-5-methyluridine

In a 100 mL stainless steel pressure reactor, ethylene glycol (20 mL) was slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring. Hydrogen gas rapidly evolved. Once the rate of bubbling subsided, 2,2'-anhydro-5-methyluridine (1.0 g, 0.4.2 mmol) and sodium bicarbonate (3 mg) were added and the reactor was sealed. The reactor was placed in an oil bath and heated to 150 °C internal temperature for 72 hours. The bomb was cooled to room temperature and opened. TLC revealed that 65% of the starting material was gone (starting material and product Rf 0.25 and 0.40 respectively in 4:1 ethyl acetate/methanol on silica gel). The reaction was worked up incomplete. The crude solution was concentrated (1 mm Hg at 100 °C, coevaporated with methanol (50 mL), boiling water (15 mL) and absolute ethanol (2x25 mL) and the residue was dried to 1.3 g of off-white foam (1 mm Hg, 25 °C, 2 hours). NMR of the crude product was consistent with 65% desired product and 35% starting material. The TLC Rf matched (on cospot) the same product generated by treating the DMT derivative above with dilute hydrochloric acid in methanol as well as the Rf of one of the spots generated by treating a sample of this product with dimethoxytrityl chloride matched the known DMT derivative (other spots were DMT on side chain and bis substituted product).

EXAMPLE 23

N4-benzoyl-2'-O-(2-phthalimido-N-oxyethyl)-5'-O-(4,4'-dimethoxytrityl)cytidine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (25, Figure 6)

The 2'-O-aminooxyethyl cytidine and guanosine analogs may be prepared via similar chemistry in combination with reported literature procedures. Key to the synthetic routes is the selective 2'-O-alkylation of unprotected nucleosides. (Guinosso, C. J., Hoke, G. D., Frier, S., Martin, J. F., Ecker, D. J., Mirabelli, C. K., Crooke, S. T., Cook, P. D., *Nucleosides Nucleotides*, 1991, *10*, 259; Manoharan, M.,

Guinosso, C. J., Cook, P. D., *Tetrahedron Lett.*, 1991, *32*, 7171; Izatt, R. M., Hansen, L. D., Rytting, J. H., Christensen, J. J., *J. Am. Chem. Soc.*, 1965, *87*, 2760. Christensen, L. F., Broom, A. D., *J. Org. Chem.*, 1972, *37*, 3398. Yano, J., Kan, L. S., Ts'o, P.O.P., *Biochim. Biophys. Acta* , 1980, *629*, 178; Takaku, H., Kamaike, K., *Chemistry Lett.* 1982, 189). Thus, cytidine may be selectively alkylated to afford the intermediate 2'-O-(2-ethylacetyl)cytidine **22**. The 3'-isomer of **22** is typically present in a minor amount and can be resolved by chromatography or crystallization. Compound **22** can be protected to give 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)cytidine (**23**). Reduction of the ester **23** should yield 2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)cytidine (**24**) which can be N-4-benzoylated, the primary hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may be phosphitylated as usual to yield N4-benzoyl-2'-O-(2-phthalimido-N-oxyethyl)-5'-O-(4,4'-dimethoxytrityl)cytidine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (**25**).

EXAMPLE 24

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (31)

In a similar fashion the 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside(. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin).)to provide 2'-O-(2-ethylacetyl)diaminopurine riboside **26** along with a minor amount of the 3'-O-isomer. Compound **26** may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine **27** by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., *PCT Int. Appl.*, 85 pp.; PIXXD2; WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine **28** and 2N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine **29** which may be reduced to

provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine (30). As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (31).

EXAMPLE 25

N-(1-hydroxyphthalimido)-5-hexene (32, Figure 9)

To a stirred solution of 5-hexane-1-ol (20 g, 0.2 mol) in THF (500 mL) was added triphenylphosphine (80 g, 0.3 mol) and N-hydroxyphthalimide (49 g, 0.3 mol). The mixture was cooled to O °C and diethylazido carboxylate (48 mL, 0.3 mol) was added slowly over a period of 1 hour. The reaction mixture was allowed to warm to room temperature and the yellow solution was stirred overnight. The solvent was then evaporated to give a yellow oil. The oil was dissolved in CH_2Cl_2 and washed with water, saturated $NaHCO_3$ solution followed by a saturated NaCl solution. The organic layer was concentrated in vacuo and the resulting oil was dissolved in a solution of CH_2Cl_2/ether to crystallize out $Ph_3P=O$ as much as possible. After three steps of purification the title compound was isolated as a yellow waxy solid (yield 93%). ^{13}C NMR: δ 21.94, 24.83, 27.58, 33.26,. 78.26, 114.91, 123.41, 128.40, 128.54, 128.63, 134.45 and 163.8 ppm.

EXAMPLE 26

N-(1-hydroxyphthalimido-5,6-hexane-diol) (33, Figure 9)

Compound 32 (2.59 g, 10 mmol), aqueous osmium tetroxide (0.15 M, 3.6 mL, 0.056 mmol) and N-methylmorpholine-N-oxide (2.46 g, 21 mmol) were dissolved in THF (100 mL). The reaction mixture was covered with aluminum foil and stirred at 25 °C for 4 hours. Tlc indicated the diol was formed. The solvent was evaporated and the residue was partitioned between water and CH_2Cl_2. The organic layer was washed with a

saturated solution of NaCl and dried over anhydrous $MgSO_4$. Concentration of the organic layer resulted in a brownish oil that was characterized by ^{13}C NMR and used in the next step without further purification. ^{13}C NMR: δ 21.92, 28.08, 32.62, 66.76, 71.96, 78.33, 123.43, 128.47, 128.71, 131.93, 132.13, 134.49, 163.89.

EXAMPLE 27

N-1-hydroxy phthalimido-6-O-dimethyoxytrityl-5,6 hexane-diol (34, Figure 9)

The product from the previous step (3.0 g) was coevaporated with pyridine (2 x 20 mL) and dissolved in pyridine (100 mL). Dimethyoxytrityl chloride (3.5 g, 10 mmol) was dissolved in of pyridine (30 mL) and added to the diol dropwise over a period of 30 minutes. After 4 hours, the reaction was quenched with methanol (10 mL). The solvent was evaporated and the residual product portioned between saturated sodium bicarbonate solution and CH_2Cl_2 (100 mL each). The organic phase was dried over anhydrous $MgSO_4$, concentrated and the residue was subjected to silica gel flash column chromatography using hexanes-ethyl acetate-triethyl amine (60:39:1). The product containing fractions were combined, concentrated in vacuo and dried to give a yellow foamy solid. NMR analysis indicated the title compound as a pure homogenous dimethyoxytritylated solid (5.05 g, 83% yield).

EXAMPLE 28

(35, Figure 9)

Compound 34 was phosphitylated (1.5 g, 2.5 mmol) in CH_2Cl_2 solvent (20 mL) by the addition of diisoproylamine tetrazolide (214 mg, 1.25 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (1.3 mL, 4.0 mmol). After stirring the solution overnight the solvent was evaporated and the residue was applied to silica column and eluted with hexanes-ethyl acetate-triethylamine (50:49:1). Concentration of the appropriate fractions gave 1.61 g of the phosphitylated compound as a yellow foam (81%).

EXAMPLE 29

Attachment of O-N linker to CPG (36, Figure 10)

Succinylated and capped CPG was prepared according to method described by P. D. Cook et al. (U.S. Patent 5,541,307). Compound 34 (0.8 mmol), dimethylaminopyridine (0.2 mmol), 2.0 g of succinylated and capped CPG triethylamine (160 μL) and DEC (4.0 mmol) were shaken together for 24 hours. Pentachlorophenyl (1.0 mmol) was then added and the resulting mixture was shaken for 24 hours. The CPG beads were filtered off and washed thoroughly with pyridine (30 mL) dichloromethane (2 x 30 mL), CH_3OH (30 mL) in ether. The CPG solid support was dried over P_2O_5 and its loading was determined to be 28 μmols/g.

EXAMPLE 30

synthesis of oligonucleotides using ON linker

The following oligonucleotides were synthesized using compound 35:

SEQ ID NO:1 5′ XTTTTTTTTTT 3′

SEQ ID NO:2 5′ X TGC ATC CCC CAG GCC ACC ATT TTT T 3′

These oligonucleotides were synthesized as phosphorothioates. Compound 35 was used as a 0.1 M solution in CH_3CN. The coupling efficiency of ON-linker was >95% as shown by trityl colors. The oligonucleotides were retained in the solid support for solid phase conjugation.

EXAMPLE 31

Conjugation of pyrene to oligonucleotides using ON-linker

Oligonucleotide SEQ ID NO:1 in CPG (1 μmol) was taken in a glass funnel reactor and of 5% methylhydrazine (5 mL) in 9:1 CH_2Cl_2/CH_3OH was added. The reactor was shaken for 30 minutes. The methyl hydrazine was drained, washed with CH_2Cl_2 and the methyl hydrazine reaction was repeated. The beads were washed with CH_2Cl_2 followed by ether and dried. Pyrene butyric acid-N-hydroxy succinimide (110 mg) in DMF (5 mL) was added. After shaking for 2 hours, the pyrene butyrate solution was drained, the oligonucleotide was deprotected in NH_4OH for 30

minutes at room temperature. The aqueous solution was then filtered and an HPLC analysis was run. The product peak had a retention time of 34.85 minutes and the diode-array spectrophotometer showed pyrene absorption.

Example 32

Conjugation of pyrene butyraldehyde to oligonucleotide (SEQ ID NO:2)

Pyrene butyraldehyde is added to SEQ ID NO:2 after $MeNHNH_2$ treatment. $NaCNBH_3$ in MeOH was then added. Deprotection of CPG followed by NH_4OH cleaving of CPG showed pyrene conjugation to oligonucleotide.

EXAMPLE 33

To a stirred solution of 1,6-hexane-diol N-hydroxy-phthalimide (6.525 g, 0.039 mol) and triphenylphosphine (10.2 g, 0.039 mol) in anhydrous THF (100 mL) was added diethylazidocarboxylate (DEAD, 7.83 g, 0.045 mol) over a period of 1 hour at 5 °C under an atmosphere of argon. The reaction mixture was then stirred at room temperature overnight. The bright yellow solution was concentrated under vacuum to remove the THF and portioned between CH_2Cl_2 and water. The organic layer was then washed with saturated $NaHCO_3$ followed by saturated NaCl. It was then dried over anhydrous $MgSO_4$ and applied to a silica column and eluted with EtOAC/hexane 1:1 to give 9.8g. The material was contaminated with $Ph_3P=O$ and was recrystallized with CH_2Cl_2/ether.

EXAMPLE 34

(Figures 11 and 12)

5-hexene-1-ol is sylylated using imidazole/TBDPS-Cl in CH_2Cl_2 to give compound 37. Compound 37 is then dihydroxylated with OSO_4/NMMO as in (Example 25 for Compound 33) to give compound 38. Compound 38 is dimethoxytritylated at the primary alcohol function to give compound 39. It is then subjected to Mitsunobu reaction with N-hydroxy-phthalimide to give compound 40. Compound 40 is then disilylated with TBAF

(tetrabutyl ammonium fluoride, 1M in THF) to give compound **41**. Compound **41** is then derivatized to a phosphoramidite **42**. Compound **41** was also separately connected to controlled pore glass beads (Compound 43).

EXAMPLE 35

2,6,9-(β-D-ribofuranosyl) purine (5.64 g, 20 mmol) was added to a suspension of 800 mg of 60% sodium hydride in oil previously washed with hexanes in 100 mL of DMF under argon. After 1 hour of stirring at room temperature allyl bromide (2 mL, 1.1 equivalent) was added to the solution and stirred at room temperature overnight. The reaction mixture was evaporated and applied to a silica column and eluted with CH_2Cl_2/CH_3OH (20:1) containing 1% triethylamine. The total yield of 2' and 3' O-allyl compounds was 5.02 g (77%). The mixture of 2' and 3' isomers was then exocyclic amine protected by treatment of DMF DMA in MeoH in quantitative yield. This material was then 5'-O-dimethyoxytritylated to give a mixture of 5'-O-dimethoxytrityl-N-2-formamidine-2'-O-(2-hydroxy ethyl)-guanosine and 5'-O-dimethoxytrityl-N2-formamidine-3'-O-(2-hydroxyethyl) guanosine in 2:1 ratio. The final compounds were purified by silica gel flash column chromatography.

EXAMPLE 36

2,6 diamino-9-(b-D-ribofuranosyl) purine (282 mg, 1 mmol) was added to a suspension of 40 mg of 60% sodium hydride in oil previously washed with hexanes in anhydrous DMF (5 mL). To this solution of 2-(bromoethoxy)-t-butyl-dimethyl silane (220 mL) was added . The mixture was stirred at room temperature overnight. The reaction mixture was evaporated and the resulting oil was partitioned between water and ethylacetate. The organic layer was dried over Na_2SO_4. The reaction mixture was purified to give the 2' and 3' isomers over the silica gel. The 2'-material was then amine protected with DMF DMA and 5'-dimethoxytrilated to give 5'-O-dimethoxytrityl-N2-formamidine-2'-O-(2-TBDMS-hydroxyethyl) guanine.

EXAMPLE 37

Oligonucleotide Synthesis

Unsubstituted and substituted oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. For phosphorothioate oligonucleotides, the standard oxidation bottle is replaced by 0.2 M solution of 3*H*-1,2-benzodithiole-3-one-1,1-dioxide in acetonitrile for the step wise thiation of the phosphite linkages. The thiation wait step is increased to 68 sec and is followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hours), the oligonucleotides are purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Analytical gel electrophoresis is accomplished in 20% acrylamide, 8 M urea, 454 mM Tris-borate buffer, pH=7.0. Oligonucleotides and phosphorothioates are judged, based on polyacrylamide gel electrophoresis, to be greater than 80% full-length material.

EXAMPLE 38

General procedure for the attachment of 2'-deoxy-2'-substituted 5'-dimethoxytriphenylmethyl ribonucleosides to the 5'-hydroxyl of nucleosides bound to CPG support.

The 2'-deoxy-2'-substituted nucleoside that will reside at the terminal 3'-position of the oligonucleotide is protected as a 5'-DMT group (the cytosine and adenine exocyclic amino groups are benzoylated and the guanine amino is isobutrylated) and treated with trifluoroacetic acid/bromoacetic acid mixed anhydride in pyridine and dimethylaminopyridine at 50°C for five hours. The solution is then evaporated under reduced pressure to a thin syrup which is dissolved in ethyl acetate and passed through a column of silica gel. The homogenous fractions are collected and evaporated to dryness. A solution of 10 mL of acetonitrile, 10 μM of the 3'-O-bromomethylester-modified nucleoside, and 1 mL of pyridine/dimethylaminopyridine (1:1) is syringed slowly (60 to 90 sec) through a 1 μM column of CPG thymidine (Applied

Biosystems, Inc.) that had previously been treated with acid according to standard conditions to afford the free 5'-hydroxyl group. Other nucleoside-bound CPG columns may be employed. The eluent is collected and syringed again through the column. This process is repeated three times. The CPG column is washed slowly with 10 mL of acetonitrile and then attached to an ABI 380B nucleic acid synthesizer. Oligonucleotide synthesis is now initiated. The standard conditions of concentrated ammonium hydroxide deprotection that cleaves the thymidine ester linkage from the CPG support also cleaves the 3',5' ester linkage connecting the pyrimidine modified nucleoside to the thymidine that was initially bound to the CPG nucleoside. In this manner, any 2'-substituted nucleoside or generally any nucleoside with modifications in the heterocycle and/or sugar can be attached at the 3' end of an oligonucleotide.

EXAMPLE 39

Modified oligonucleotide synthesis for incorporation of 2'-substituted nucleotides

A. ABI Synthesizer

Oligonucleotide sequences incorporating 1-[2'-O-(2-phthalimido-N-oxyethyl)-5'-O-(4,4'-dimethoxytrityl)-β-D-ribofuranosyl]thymine were synthesized on an ABI 380B utilizing phosphoramidite chemistry with double-coupling and increased coupling times (5 and 10 min). The 2'-O-aminooxyethoxy phosphoramidite was used at a starting concentration of 0.08 M and 10% tert-butyl peroxide in acetonitrile was used as the oxidizer. Deoxy phosphoramidites were used at 0.2 M. Final concentrations of 2'-O-aminooxyethoxy and deoxy amidites were 0.04 M and 0.1 M, respectively. The oligonucleotides were cleaved from the CPG support and the base protecting groups removed using concentrated ammonia at 55°C for 6 hours. The oligonucleotides were purified by size exclusion chromatography over Sephadex G25 and analyzed by electrospray mass spectrometry and capillary gel electrophoresis. Deoxy phosphoramidites were purchased from Perseptive Biosystems GmbH.

(SEQ ID NO:6) CTC GTA CCt TTC CGG TCC. LRMS (ES-) *m/z* : calcd: 5453.2; found: 5453.5.

(SEQ ID NO:8) CTC GTA Ctt ttC CGG TCC. LRMS (ES-) *m/z* : calcd: 5693.2; found: 5692.9.

(SEQ ID NO:12) GCG ttt ttt ttt tGC G. LRMS (ES-) *m/z* : calcd: 5625.7; found: 5625.9.

B. Expedite Synthesizer

Oligonucleotides incorporating 1-[2'-O-(2-phthalimdo-N-oxyethyl-5'-O-dimethoxytrityl-B-D-ribofuramosyl)-6-N-benzoyl-thymine were synthesized in an Expedite 8690 Synthesizer. 130 mg of the amidite was dissolved in dry CH_3CN (1.3 mL, app. 0.08M). 10% t-BuOOH in CH_3CN v/v was used as the oxidizing agent. An extended coupling and waiting times were used and a 10 min. oxidation was employed. The oligonucleotide synthesis revealed excellent coupling yields (>98%). Oligonucleotides were purified and their mass spec and profiles determined.

Oligonucleotide	Sequence	SEQ ID NO:
V	CTC GTA CCa TTC CGG TCC	13
VI	GGa CCG Gaa GGT aCG aG	14
VII	aCC GaG GaT CaT GTC GTa CGC	15

where a represents 1-[2'-O-(2-aminooxyethyl)-β-D-ribofuranosyl]adenosine.

EXAMPLE 40

Oligonucleotide Having 2'-Substituted Oligonucleotides Regions Flanking Central 2'-Deoxy Phosphorothioate Oligonucleotide Region

A 15mer RNA target of the sequence 5'GCGTTTTTTTTTGCG 3' (SEQ ID NO:3) is prepared in the normal manner on the DNA sequencer using RNA protocols. A series of complementary phosphorothioate oligonucleotides having 2'-O-substituted nucleotides in regions that flank a 2'-deoxy region are prepared utilizing 2'-O-substituted nucleotide precursors prepared as per known literature preparations, *i.e.* 2'-O-

methyl, or as per the procedure of International Publication Number WO 92/03568, published March 5, 1992. The 2'-O-substituted nucleotides are added as their 5'-O-dimethoxytrityl-3'-phosphoramidites in the normal manner on the DNA synthesizer. The complementary oligonucleotides have the sequence of 5' CGCAAAAAAAAAAAACGC 3' (SEQ ID NO:4). The 2'-O-substituent is located in CGC and CG regions of these oligonucleotides. The 2'-O-substituents used are 2'-aminooxyethyl, 2'-O-ethylaminooxyethyl and 2'-O-dimethylaminooxyethyl.

EXAMPLE 41

Hybridization Analysis.

A. Evaluation of the thermodynamics of hybridization of 2'-modified oligonucleotides.

The ability of the 2'- modified oligonucleotides to hybridize to their complementary RNA or DNA sequences is determined by thermal melting analysis. The RNA complement is synthesized from T7 RNA polymerase and a template-promoter of DNA synthesized with an Applied Biosystems, Inc. 380B RNA species is purified by ion exchange using FPLC (LKB Pharmacia, Inc.). Natural antisense oligonucleotides or those containing 2'-modifications at specific locations are added to either the RNA or DNA complement at stoichiometric concentrations and the absorbance (260 nm) hyperchromicity upon duplex to random coil transition is monitored using a Gilford Response II spectrophotometer. These measurements are performed in a buffer of 10 mM Na-phosphate, pH 7.4, 0.1 mM EDTA, and NaCl to yield an ionic strength of 10 either 0.1 M or 1.0 M. Data is analyzed by a graphic representation of $1/T_m$ vs ln(Ct), where (Ct) was the total oligonucleotide concentration. From this analysis the thermodynamic para-meters are determined. Based upon the information gained concerning the stability of the duplex of heteroduplex formed, the placement of modified pyrimidine into oligonucleotides are assessed for their effects on helix stability. Modifications that drastically alter the stability of the hybrid exhibit reductions in the free energy (delta G)

and decisions concerning their usefulness as antisense oligonucleotides are made.

As is shown in the following table (Table 1), the incorporation of 2'-substituted nucleosides of the invention into oligonucleotides can result in significant increases in the duplex stability of the modified oligonucleotide strand (the antisense strand) and its complementary RNA strand (the sense strand). The stability of the duplex increased as the number of 2'-substituted nucleosides in the antisense strand increased. As is evident from Table 1 the addition of a 2'-substituted nucleoside, irrespective of the individual nucleoside or the position of that nucleoside in the oligonucleotide sequence, resulted in an increase in the duplex stability.

In Table 1, the small case nucleosides represent nucleosides that include substituents of the invention. Effects of 2'-O-aminooxyethoxy modifications on DNA(antisense) - RNA(sense) duplex stability.

Table 1

SEQ ID NO:	Sequence
5	CTC GTA CCT TTC CGG TCC
6	CTC GTA CCt TTC CGG TCC
7	CTC GTA CTT TTC CGG TCC
8	CTC GTA Ctt ttC CGG TCC
9	GCG TTT TTT TTT TGC G
10	GCG ttt ttt ttt tGC G
11	GCG TTT TTT TTT TGC G*
12	GCG ttt ttt ttt tGC G*
13	CTC GTA CCa TTC CGG TCC
14	GGa CCG Gaa GGT aCG aG
15	aCC GaG GaT CaT GTC GTa CGC

t = 1-[2'-O-(2-aminooxyethyl)-β-D-ribofuranosyl]thymine. a = 1-[2'-O-(2-aminooxyethyl)-β-D-ribofuranosyl]adenosine. * = was hybridized against DNA as sense strand.

SEQ ID NO:	subs.	T_m °C	ΔT_m °C	ΔT_m °C/sub.
5	0	65.2 ±0.0		
6	1	64.8 ±0.1	-0.5 ±0.1	-0.5 ±0.1
7	0	61.5 ±0.0		
8	4	65.6 ±0.4	4.1 ±0.4	1.0 ±0.1
9	0	48.2 ±0.6		
10	10	60.0 ±0.0	11.9 ±0.7	1.19 ±0.07
11	0	53.5 ±0.1†		
12	10	44.0 ±0.2†	-9.4 ±0.3†	-0.94 ±0.03†

As is evident from Table 1, the duplexes formed between RNA and oligonucleotides containing 2'-substituents of the invention exhibited increased binding stability as measured by the hybridization thermodynamic stability. While we do not wish to be bound by theory, it is presently believed that the presence of a 2'-substituent of the invention results in the sugar moiety of the 2'-substituted nucleoside assuming substantially a 3'-endo conformation and this results in the oligonucleotide-RNA complex assuming an A-type helical conformation.

EXAMPLE 42

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridie (101)

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and sat'd sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline

product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

EXAMPLE 43

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (102)

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product. NMR (DMSO-*d6*) d 1.05 (s, 9H, t-butyl), 1.45 (s, 3 H, CH3), 3.5-4.1 (m, 8 H, CH2CH2, 3'-H, 4'-H, 5'-H, 5"-H), 4.25 (m, 1 H, 2'-H), 4.80 (t, 1 H,

CH2O-H), 5.18 (d, 2H, 3'-OH), 5.95 (d, 1 H, 1'-H), 7.35-7.75 (m, 11 H, Ph and C6-H), 11.42 (s, 1 H, N-H).

EXAMPLE 44

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (103)

Nucleoside **102** (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get **103** as white foam (21.819, 86%). Rf 0.56 (ethyl acetate:hexane, 60:40). MS (FAB^-)m/e 684 ($M-H^+$)

EXAMPLE 45

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (104)

Compound **103** (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 hr the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1eg.) was added and the mixture for 1 hr. Solvent removed under vacuum; residue chromatographed to get compound **104** as

white foam (1.95, 78%). Rf 0.32 (5% MeOH in CH_2Cl_2). MS (Electrospray$^-$) m/e 566 ($M-H^\oplus$)

EXAMPLE 46

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (105)

Compound **104** (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodiumcyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 hr, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase dried over anhydrous Na_2SO_4, evaporated to dryness. Residue dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodiumcyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4; and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get **105** as a white foam (14.6g, 80%). Rf 0.35 (5% MeOH in CH_2Cl_2). MS ($FAB^\oplus$) m/e 584 ($M+H^\oplus$)

EXAMPLE 47

2'-O-(dimethylaminooxyethyl)-5-methyluridine (106)

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry,

kept over KOH). This mixture of triethylamine-2HF was then added to compound **105** (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get **106** (766mg, 92.5%). Rf 0.27 (5% MeOH in CH_2Cl_2). MS (FAB$^{\oplus}$) m/e 346 (M+H$^{\oplus}$)

EXAMPLE 48

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (107)

Compound **106** (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH2Cl2 (containing a few drops of pyridine) to get **107** (1.13g, 80%). Rf 0.44 ((10% MeOH in CH_2Cl_2). MS (FAB$^{\oplus}$) m/e 648 (M+H$^{\oplus}$)

EXAMPLE 49

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (108)

Compound **107** (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^{i},N^{i}-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The

progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get **108** as a foam (1.04g, 74.9%). Rf 0.25 (ethyl acetate:hexane, 1:1). ^{31}P NMR ($CDCl_3$) δ 150.8 ppm; MS (FAB®) m/e 848 ($M+H^®$)

EXAMPLE 50

2'/3'-O-allyl adenosine (109)

Adenosine (20g, 74.84mmol) was dried over P_2O_5 under high vacuum at 40°C for two days. It was then suspended in DMF under inert atmosphere. Sodium hydride (2.5g, 74.84mmol, 60% dispersion in mineral oil), stirred at room temperature for 10 minutes. Then allyl bromide (7.14mL, 82.45mmol) was added dropwise and the reaction mixture was stirred at room temperature overnight. DMF was removed under vacuum and residue was washed with ethyl acetate (100mL). Ethyl acetate layer was decanted. Filtrate obtained contained product. It was then placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get **109** (15.19g, 66%). Rf 0.4, 0.4a ((10% MeOH in CH_2Cl_2)

EXAMPLE 51

2'/3'-O-allyl-N^6-benzoyl adenosine (110)

Compound **109**(15.19g, 51.1mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then dissolved in anhydrous pyridine (504.6mL) under inert atmosphere. Trimethylchlorosilane (32.02mL, 252.3mmol) was added at 0°C and the reaction mixture was stirred for 1 hr under inert atmosphere. Then benzoyl chloride (29.4mL, 252.3mmol) was added dropwise. Once the addition of benzoyl chloride was over, the reaction mixture was brought to room temperature and stirred for 4 hrs. Then the reaction mixture was brought to

0°C in an ice bath. Water (100.9mL). was added and the reaction mixture was stirred for 30 minutes. Then NH_4OH (100.0mL, 30% aqueous solution w/w) was added, keeping the reaction mixture at 0°C and stirring for an additional 1 hr. Solvent evaporated residue partitioned between water and ether. Product precipitates as an oil, which was then chromatographed (5%MeOH in CH_2Cl_2) to get **13** as a white foam (12.67g, 62%).

EXAMPLE 52

3'-O-allyl-5'-O-tert-butyldiphenylsilyl-N^6-benzoyl-adenosine (111)

Compound **110** (11.17g, 27.84mmol) was dried over P_2O_5 under vacuum at 40°C, then dissolved in dry CH_2Cl_2 (56mL, sure seal from Aldrich). 4-dimethylaminopyridine (0.34g, 2.8mmol), triethylamine (23.82mL, 167mmol) and t-butyldiphenylsilyl chloride were added. The reaction mixture was stirred vigorously for 12 hr. Reaction was monitored by TLC (ethyl acetate:hexane 1:1). It was then diluted with CH_2Cl_2 (50mL) and washed with water (3x30mL). Dichloromethane layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. Residue purified by flash chromatography (ethyl acetate:hexane 1:1 as eluent) to get **111** as a white foam (8.85g, 49%). Rf 0.35 (ethyl acetate:hexane, 1:1)

EXAMPLE 53

5'-O-tert-butyldiphenylsilyl-N^6-benzoyl-2'-O-(2,3-dihydroxypropyl)-adenosine (112)

Compound **111** (5.5g, 8.46mmol), 4-methylmorpholine N-oxide (1.43g, 12.18mmol) were dissolved in dioxane (45.42mL). 4% aqueous solution of OSO_4 (1.99mL, 0.31mmol) was added. The reaction mixture was protected from light and stirred for 3 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Ethyl acetate (100mL) was added and the resulting reaction mixture was washed with water (1x50mL). Ethyl acetate layer was dried

over anhydrous Na_2SO_4 and evaporated to get **112** (5.9g) and used for next step without purification. Rf 0.17 (5% MeOH in CH_2Cl_2)

EXAMPLE 54

5'-O-*tert*-butyldiphenylsilyl-N^6-benzoyl-2'-O-(formylmethyl)-adenosine (113)

Compound **112** (5.59g, 8.17mmol) was dissolved in dry CH_2Cl_2 (40.42mL). To this $NaIO_4$ adsorbed on silica gel (Ref. *J. Org. Chem.* 1997, 62, 2622-2624) (16.34g, 2g/mmol) was added and stirred at ambient temperature for 30 minutes. Reaction monitored by TLC (5% MeOH in CH_2Cl_2). Reaction mixture was filtered and the filtrate washed thoroughly with CH_2Cl_2. Dichloromethane layer evaporated to get the aldehyde **113** (5.60g) that was used in the next step without purification. Rf 0.3 (5% MeOH in CH_2Cl_2)

EXAMPLE 55

5'-O-*tert*-butyldiphenylsilyl-N^6-2'-O-(2-hydroxyethyl) adenosine (114)

Compound **113** (5.55g, 8.50mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate in anhydrous MeOH (85mL). Reaction mixture was protected from moisture. Sodiumcyanoborohydride (1.08g, 17.27mmol) was added and reaction mixture stirred at ambient temperature for 5 hrs. The progress of the reaction was monitored by TLC (5% MeOH in CH_2Cl_2). The reaction mixture was diluted with ethyl acetate (150mL), then washed with 5% $NaHCO_3$ (75mL) and brine (75mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. Residue purified by flash chromatography (5% MeOH in CH_2Cl_2) to get **114** (4.31g, 77.8%). Rf 0.21 (5% MeOH in CH_2Cl_2). MS (FAB®) m/e 655 ($M+H^®$), 677 ($M+Na^®$)

EXAMPLE 56

5'-tert-butyldiphenylsilyl-N^6-benzoyl-2'-O-(2-phthalimidooxyethyl) adenosine (115)

Compound **114** (3.22g, 4.92mmol) was mixed with triphenylphosphine (1.55g, 5.90mmol) and N-hydroxyphthalimide (0.96g, 5.90mmol). It was then dried over P_2O_5 under vacuum at 40°C for two days. Dissolved dried mixture in anhydrous THF (49.2mL) under inert atmosphere. Diethyl azodicarboxylate (0.93mL, 5.90mmol) was added dropwise. The rate of addition was maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was completed, the reaction was stirred for 4 hrs, monitored by TLC (ethylacetate:hexane 70:30). Solvent was removed under vacuum and the residue dissolved in ethyl acetate (75mL). The ethyl acetate layer was washed with water (75mL), then dried over Na_2SO_4, concentrated and chromatographed (ethylacetate:hexane 70:30) to get **115** (3.60g, 91.5%). Rf 0.27 ethyl acetate:hexane, 7:3) MS (FAB$^{\oplus}$) m/e 799 ($M+H^{\oplus}$), 821 ($M+Na^{\oplus}$)

EXAMPLE 57

5'-O-tert-butyldiphenylsilyl-N^6-benzoyl-2'-O-(2-formaldoximinooxyethyl) adenosine (116)

Compound **115** (3.5g, 4.28mmol) was dissolved in CH_2Cl_2 (43.8mL). N-methylhydrazine (0.28mL, 5.27mmol) was added at -10°C and the reaction mixture was stirred for 1 hr at -10 to 0°C. Reaction monitored by TLC (5% MeOH in CH_2Cl_2). A white precipitate formed was filtered and filtrate washed with ice cold CH_2Cl_2 thoroughly. Dichloromethane layer evaporated on a rotavapor keeping the water bath temperature of rotavapor at less than 25°C. Residue obtained was then dissolved in MeOH (65.7mL). Formaldehyde (710mL, 4.8 mmol, 20% solution in water) was added and the reaction mixture was stirred at ambient temperature for 1 hr. Reaction monitored by ^1H NMR. Reaction mixture concentrated and chromatographed (5% MeOH in

CH_2Cl_2) to get **116** as a white foam (2.47g, 83%). Rf 0.37 (5% MeOH in CH_2Cl_2). MS (FAB$^{\oplus}$) m/e 681 ($M+H^{\oplus}$)

EXAMPLE 58

5'-tert-butyldiphenylsilyl-N^6-benzoyl-2'-O-(2-N,N-dimethylaminooxyethyl) adenosine (117)

Compound **116** (2.2g, 3.23mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in MeOH (32mL). Reaction protected from moisture. Sodium cyanoborohydride (0.31g) was added at 10°C and reaction mixture was stirred for 10 minutes at 10°C. It was then brought to ambient temperature and stirred for 2 hrs, monitored by TLC (5% MeOH in CH_2Cl_2). 5% aqueous sodiumbicarbonate (100mL) and extracted with ethyl acetate (3x50mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (32mL). Formaldehyde (0.54mL, 3.55mmol, 20% aqueous solution) was added and stirred at room temperature for 10 minutes. Sodium cyanoborohydride (0.31g) was added at 10°C and stirred for 10 minutes at 10°C. Then the reaction mixture was removed from ice bath and stirred at room temperature for an additional 2 hrs, monitored by TLC (5% MeOH in CH_2Cl_2). Reaction mixture was diluted with 5% aqueous $NaHCO_3$ (100mL) and extracted with ethyl acetate (3x50mL). Ethyl acetate layer was dried, evaporated and chromatographed (5% MeOH in CH_2Cl_2) to get **117** (1.9g, 81.8%). Rf 0.29 (5% MeOH in CH_2Cl_2). MS (FAB$^{\oplus}$) m/e 697 ($M+H^{\oplus}$), 719 ($M+Na^{\oplus}$)

EXAMPLE 59

N^6-benzoyl-2'-O-(N,N-dimethylaminooxyethyl) adenosine (118)

To a solution of Et_3N-3HF (1.6g, 10mmol) in anhydrous THF (10mL) triethylamine (0.71mL, 5.12mmol) was added. Then this mixture was added to compound **117** (0.72g, 1mmol) and stirred at room temperature under inert atmosphere for 24 hrs.

Reaction monitored by TLC (10% MeOH in CH_2Cl_2). Solvent removed under vacuum and the residue chromatographed (10% MeOH in CH_2Cl_2) to get **118** (0.409g, 89%). Rf 0.40 (10% MeOH in CH_2Cl_2). MS (FAB$^{\oplus}$) m/e 459 ($M+H^{\oplus}$)

EXAMPLE 60

5'-O-dimethoxytrityl-N^6-benzoyl-2'-O-(2-N,N-dimethylaminooxyethyl) adenosine (119)

Compound **118** (0.4g, 0.87mmol) was dried over P_2O_5 under vacuum overnight at 40°C. 4-dimethylaminopyridine (0.022g, 0.17mmol) was added. Then it was co-evaporated with anhydrous pyridine (9mL). Residue was dissolved in anhydrous pyridine (2mL) under inert atmosphere, and 4,4'-dimethoxytrityl chloride (0.58g, 1.72mmol) was added and stirred at room temperature for 4 hrs. TLC (5% MeOH in CH_2Cl_2) showed the completion of the reaction. Pyridine was removed under vacuum, residue dissolved in CH_2Cl_2 (50mL) and washed with aqueous 5% $NaHCO_3$ (30mL) solution followed by brine (30mL). CH_2Cl_2 layer dried over anhydrous Na_2SO_4 and evaporated. Residue chromatographed (5% MeOH in CH_2Cl_2 containing a few drops of pyridine) to get **119** (0.5g, 75%). Rf 0.20 (5% MeOH in CH_2Cl_2). MS (Electrospray$^-$) m/e 759 ($M+H^{\oplus}$)

EXAMPLE 61

N^6-benzoyl-5'-O-DMT-2'-O-(N,N-dimethylaminooxyethyl) adenosine-3'-O-phosphoramidite (120)

Compound **119** (0.47g, 0.62mmol) was co-evaporated with toluene (5mL). Residue was mixed with N,N-diisopropylamine tetrazolide (0.106g, 0.62mmol) and dried over P_2O_5 under high vacuum overnight. Then it was dissolved in anhydrous CH_3CN (3.2mL) under inert atmosphere. 2-cyanoethyl- tetraisopropyl phosphordiamidite (0.79mL, 2.48mmol) was added dropwise and the reaction mixture was stirred at room temperature under inert atmosphere for 6 hrs. Reaction was monitored by TLC (ethyl

acetate containing a few drops of pyridine). Solvent was removed, then residue was dissolved in ethyl acetate (50mL) and washed with 5% aqueous $NaHCO_3$ (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4, evaporated, and residue chromatographed (ethyl acetate containing a few drops of pyridine) to get **120** (0.45g, 76%). MS (Electrospray$^-$) m/e 959 ($M+H^e$). ^{31}P NMR ($CDCl_3$) δ 151.36, 150.77 ppm

EXAMPLE 62

2′/3′-O-allyl-2,6-diaminopurine riboside (121 and 122)

2,6-Diaminopurine riboside (30g, 106.4 mmol) was suspended in anhydrous DMF (540 mL). Reaction vessel was flushed with argon. Sodium hydride (3.6g, 106.4mmol, 60% dispersion in mineral oil) was added and the reaction stirred for 10 min. Allyl bromide (14.14mL, 117.22mmol) was added dropwise over 20 min. The resulting reaction mixture stirred at room temperature for 20 hr. TLC (10% MeOH in CH_2Cl_2) showed complete disappearance of starting material. DMF was removed under vacuum and the residue absorbed on silica was placed on a flash column and eluted with 10% MeOH in CH_2Cl_2. Fractions containing mixture of 2′ and 3′ allylated product was pooled together and concentrated to dryness to yield a mixture of **121** and **122** (26.38g, 77%). Rf 0.26, 0.4 (10% MeOH in CH_2Cl_2)

EXAMPLE 63

2′-O-allyl-guanosine (123)

A mixture of **121** and **122** (20g, 62.12mmol) was suspended in 100 mm sodium phosphate buffer (pH 7.5) and adenosine deaminase (1g) was added. The resulting solution was stirred very slowly for 60 hr, keeping the reaction vessel open to atmosphere. Reaction mixture was then cooled in ice bath for one hr and the precipitate obtained was filtered, dried over P_2O_5 under high vacuum to yield **123** as

white powder (13.92 g, 69.6% yield). Rf 0.19 (20% MeOH in CH_2Cl_2)

EXAMPLE 64

2'-O-allyl-3', 5'-bis(*tert*-butyl diphenylsilyl) guanosine (124)

2'-O-allyl-guanosine (6g, 18.69mmol) was mixed with imidazole (10.18g, 14.952mmol) and was dried over P_2O_5 under high vacuum overnight. It was then flushed with argon. Anhydrous DMF (50mL) was added and stirred with the reaction mixture for 10 minutes. To this *tert*-butyldiphenylsilyl chloride (19.44mL, 74.76mmol) was added and the reaction mixture stirred overnight under argon atmosphere. DMF was removed under vacuum and the residue was dissolved in ethyl acetate (100mL) and washed with water (2 x 75mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. Residue placed on a flash column and eluted with 5% MeOH in CH_2Cl_2. Fractions containing the product were pooled together and evaporated to yield **124** (10.84g, 72% yield) as a white foam. Rf = ? MS (FAB^e) m/e 800 ($M+H^e$), 822 ($M+Na^e$).

EXAMPLE 65

2'-O-(2-hydroxyethyl)-3',5'-bis(*tert*-butyldiphenylsilyl) guanosine (125)

Compound **124** (9g, 11.23mmol) was dissolved in CH_2Cl_2 (80mL). To the clear solution acetone (50 mL), 4-methyl morpholine-N-oxide (1.89g, 16.17mmol) was added. The reaction flask was protected from light. Thus 4% aqueous solution of osmium tetroxide was added and the reaction mixture was stirred at room temperature for 6 hr. Reaction volume was concentrated to half and ethyl acetate (50mL) was added. It was then washed with water (30mL) and brine (30mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4

and evaporated to dryness. Residue was then dissolved in CH_2Cl_2 and $NaIO_4$ adsorbed on silica (21.17g, 2g/mmol) was added and stirred with the reaction mixture for 30 min. The reaction mixture was filtered and silica was washed thoroughly with CH_2Cl_2. Combined CH_2Cl_2 layer was evaporated to dryness. Residue was then dissolved in dissolved in 1M pyridinium-p-toluene sulfonate (PPTS) in dry MeOH (99.5mL) under inert atmosphere. To the clear solution sodium cyanoborohydride (1.14g, 18.2mmol) was added and stirred at room temperature for 4 hr. 5% aqueous sodium bicarbonate (50mL) was added to the reaction mixture slowly and extracted with ethyl acetate (2 x 50mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. Residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to yield **125** (6.46 g, 72% yield). MS (Electrospray-) m/e 802 ($M-H^{\oplus}$)

EXAMPLE 66

2'-O-[(2-phthalimidoxy)ethyl]-3',5'-bis (tert butyldiphenylsilyl) guanosine (126)

Compound **125** (3.7g, 4.61mmol) was mixed with Ph_3P (1.40g, 5.35mmol), and hydroxy phthalimide (0.87g, 5.35mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. These anhydrous THF (46.1mmol) was added to get a clear solution under inert atmosphere. Diethylazidocarboxylate (0.73mL, 4.61mmol) was added dropwise in such a manner that red color disappears before addition of the next drop. Resulting solution was then stirred at room temperature for 4 hr. THF was removed under vacuum and the residue dissolved in ethyl acetate (75mL) and washed with water (2 x 50mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. Residue was purified by column chromatography and eluted with 7%

MeOH in ethyl acetate to yield **126** (2.62g, 60% yield). Rf 0.48 (10% MeOH in CH_2Cl_2). MS (FAB^-) m/e 947 ($M-H^\oplus$).

EXAMPLE 67

2'-O-(2-phthalimido-N-oxyethyl)-3',5'-O-bis-tert-butyldiphenylsilyl-N2-isobutyrylguanosine (127)

2'-O-(2-phthalimido-N-oxyethyl)-3',5'-O-bis-tert-butyldiphenylsilyl guanosine (3.66 g, 3.86 mmol) was dissolved in anhydrous pyridine (40 mL), the solution was cooled to 5° C, and isobutyryl chloride (0.808 mL, 7.72 mmol) was added dropwise. The reaction mixture was allowed to warm to 25° C, and after 2h additional isobutyryl chloride (0.40 mL, 3.35 mmol) was added at 25° C. After 1h the solvent was evaporated in vaccuo (0.1 torr) at 30° C to give a foam which was dissolved in ethyl acetate (150 mL) to give a fine suspension. The suspension was washed with water (2 x 15 mL) and brine (4 mL), and the organic layer was separated and dried over $MgSO_4$. The solvent was evaporated in vaccuo to give a foam, which was purified by column chromatography using CH_2Cl_2-MeOH, 94:6, v/v, to afford the title compound as a white foam (2.57 g, 65%). ^1H NMR($CDCl_3$): d 11.97 (br s, 1H), 8.73 (s, 1H), 7.8-7.2 (m, 25H), 5.93 (d, 1H, $J_{1',2'}$ = 3.3 Hz), 4.46 (m, 1H), 4.24 (m, 2H), 3.83 (m, 2H), 3.60 (m, 2H), 3.32 (m, 1H), 2.67 (m, 1H), 1.30 (d, 3H, J = 3.2 Hz), 1.26 (d, 3H, j = 3.1 Hz), 1.05 (s, 9H), 1.02 (s, 9H).

This compound was further derivatized into the corresponding phosphoramidite using the chemistries described above for A and T analogs to give compound **128**.

EXAMPLE 68

3'-O-acetyl-2'-O-(2-N,N-dimethylaminooxyethyl)-5'-O-*tert*-butyldiphenylsilyl thymidine (129)

Compound **105** (3.04g, 5.21mmol) was dissolved in chloroform (11.4mL). To this was added dimethylaminopyridine (0.99g, 8.10mmol) and the reaction mixture was stirred for 10 minutes. Acetic anhydride (0.701 g, 6.87 mmol) was added and the reaction mixture was stirred overnight. The reaction mixture was then diluted with CH_2Cl_2 (40mL) and washed with saturated $NaHCO_3$ (30 mL) and brine (30 mL). CH_2Cl_2 layer evaporated to dryness. Residue placed on a flash column and eluted with ethyl acetate: hexane (80:20) to yield **129**. Rf 0.43 (ethyl acetate:hexane, 80:20). MS (Electrospray$^-$) m/e 624 ($M-H^\oplus$)

EXAMPLE 69

2'-O-(2-N,N-dimethylaminooxyethyl)-5'-O-*tert*-butyldiphenylsilyl 5-methyl cytidine (130)

A suspension of 1,2,4-triazole (5.86g, 84.83mmol) in anhydrous CH_3CN (49mL) was cooled in an ice bath for 5 to 10 min. under argon atmosphere. To this cold suspension $POCl_3$ (1.87mL, 20mmol) was added slowly over 10 min. and stirring continued for an additional 5 min. Triethylamine (13.91mL, 99.8mmol) was added slowly over 30 min., keeping the bath temperature around 0-2°C. After the addition was complete the reaction mixture was stirred at this temperature for an additional 30 minutes when compound **35** (3.12g, 4.99mmol) was added in anhydrous acetonitrile (3mL) in one portion. The reaction mixture was stirred at 0-2°C for 10 min. Then ice bath was removed and the reaction mixture was stirred at room temperature for 1.5 hr. The reaction mixture was cooled to °C and this was concentrated to smaller volume and dissolved in ethyl acetate (100mL), washed with water (2 x 30mL) and brine (30mL). Organic

layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. Residue obtained was then dissolved in saturated solution of NH_3 in dioxane (25mL) and stirred at room temperature overnight. Solvent was removed under vacuum. The residue was purified by column chromatography and eluted with 10% MeOH in CH_2Cl_2 to get **130**.

EXAMPLE 70

2'-O-(2,N, N-dimethylaminooxyethyl)-N^4-benzoyl-5'-O-*tert*-butyldiphenylsilylcytidine (131)

Compound **130** (2.8g, 4.81mmol) was dissolved in anhydrous DMF (12.33mL). Benzoic anhydride (1.4g, 6.17mmol) was added and the reaction mixture was stirred at room temperature overnight. Methanol was added (1mL) and solvent evaporated to dryness. Residue was dissolved in dichloromethane (50mL) and washed with saturated solution of $NaHCO_3$ (2 x 30 mL) followed by brine (30mL). Dichloromethane layer was dried over anhydrous Na_2SO_4 and concentrated. The residue obtained was purified by column chromatography and eluted with 5% MeOH in CH_2Cl_2 to yield **131** as a foam.

EXAMPLE 71

N^4-Benzoyl-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyl cytidine (132)

Compound **131** (2.5g, 3.9mmol) was dried over P_2O_5 under high vacuum. In a 100mL round bottom flask, triethylamine trihydrofluoride (6.36mL, 39mmol) is dissolved in anhydrous THF (39mL). To this, triethylamine (2.72mL, 19.5mmol) was added and the mixture was quickly poured into compound **131** and stirred at room temperature overnight. Solvent is removed under vacuum and the residue kept in a flash column and eluted with 10% MeOH in CH_2Cl_2 to yield **132**.

EXAMPLE 72

N^4-Benzoyl-2'-O-(2-N,N-dimethylaminooxyethyl)-5-O'-dimetoxytrityl-5-methyl cytidine (133)

Compound **132** (1.3g, 2.98mmol) was dried over P_2O_5 under high vacuum overnight. It was then co-evaporated with anhydrous pyridine (10mL). Residue was dissolved in anhydrous pyridine (15mL), 4-dimethylamino pyridine (10.9mg, 0.3mmol) was added and the solution was stirred at room temperature under argon atmosphere for 4 hr. Pyridine was removed under vacuum and the residue dissolved in ethyl acetate and washed with 5% $NaHCO_3$ (20mL) and brine (20mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. Residue was placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 containing a few drops of pyridine to yield compound **133**.

EXAMPLE 73

N^4-Benzoyl-2'-O-(2-N,N-dimethylaminooxyethyl)-5-dimethoxytrityl-5-methyl cytidine-3'-O-phosphoramidite (134)

Compound **133** (1.54g, 2.09mmol) was co-evaporated with toluene (10mL). It was then mixed with diisopropylamine tetrazolide (0.36g, 2.09mmol) and dried over P_2O_5 under high vacuum at 40°C overnight. Then it was dissolved in anhydrous acetonitrile (11mL) and 2-cyanoethyl-tetraisopropylphosphoramidite (2.66mL, 8.36mmol) was added. The reaction mixture was stirred at room temperature under inert atmosphere for 4 hr. Solvent was removed under vacuum. Ethyl acetate (50mL) was added to the residue and washed with 5% $NaHCO_3$ (30mL) and brine (30mL). Organic phase was dried over anhydrous Na_2SO_4 and concentrated to dryness. Residue placed on a flash column and eluted with ethylacetate:hexane (60:40) containing a few drops of pyridine to get **134**.

EXAMPLE 74

2'-O-dimethylaminooxyethyl-2,6-diaminopurine riboside phosphoramidite (135)

For the incorporation of 2'-O-dimethylaminooxyethyl-2,6-diaminopurine riboside into oligonucleotides, we elected to use the phosphoramidite of protected 6-amino-2-fluoropurine riboside **135**. Post-oligo synthesis, concomitant with the deprotection of oligonucleotide protection groups, the 2-fluoro group is displaced with ammonia to give the 2,6-diaminopurine riboside analog. Thus, 2,6-diaminopurine riboside is alkylated with dimethylaminooxyethylbromide **136** to afford a mixture of 2'-O- dimethylaminooxyethyl-2,6-diaminopurine riboside **137** and the 3'- isomer **138**. Typically after functionalizing the 5'-hydroxyl with DMT to provide 5'-O-(4,4'-dimethoxytrityl)-2'-O- dimethylaminooxyethyl-2,6-diaminopurine riboside **139**, the 2'-isomer may be resolved chromatographically. Fluorination of **139** via the Schiemann reaction (Krolikiewicz, K.; Vorbruggen, H. Nucleosides Nucleotides, **1994**, *13*, 673-678) provides 2'-O-dimethylaminooxyethyl-6-amino-2-fluoro-purine riboside **140** and standard protection protocols affords 5'-O-(4,4'-dimethoxytrityl)-2'-O- dimethylaminooxyethyl-6-dimethyformamidine-2-fluoropurine riboside **140**. Phosphitylation of **140** gives 5'-O-(4,4'-dimethoxytrityl)-2'-O- dimethylaminooxyethyl-6-dimethyformamidine-2-fluoropurine riboside-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] **138**.

In the event that compound **139** cannot be resolved chromatographically from the 3'-isomer, the mixture of compounds **137** and **138** may be treated with adenosine deaminase, which is known to selectively deaminate 2'-O-substituted adenosine analogs in preference to the 3'-O-isomer, to afford 2'-O- dimethylaminooxyethylguanosine **142**.

5'-O-(4,4'-dimethoxytrityl)-2'-O-dimethylaminooxyethylguanosine **140** may be converted to the 2,6-diaminopurine riboside analog **139** by amination of the 6-oxo group (Gryaznov, S.; Schultz, R. G. Tetrahedron Lett. 1994, 2489-2492). This was then converted to the corresponding amidite **144** by standard protection methods and protocols for phosphitylation.

EXAMPLE 75

2'/3'-O-[2-(*tert*-butyldimethylsilylhydroxy) ethyl]-2,6-diaminopurine riboside (145 and 146)

2,6-diaminopurine riboside (10g, 35.46mmol) was dried over P_2O_5 under high vacuum. It was suspended in anhydrous DMF (180mL) and NaH (1.2g, 35.46mmol, 60% dispersion in mineral oil) was added. The reaction mixture was stirred at ambient temperature at inert atmosphere for 30 minutes. To this (2-bromoethoxy)-*tert*-butyldimethylsilane (12.73 g, 53.2mmol) was added dropwise and the resulting solution was stirred at room temperature overnight. DMF was removed under vacuum, residue was dissolved in ethyl acetate (100mL) and washed with water (2 x70mL). Ethyl acetate layer was dried over anhydrous $MgSO_4$ and concentrated to dryness. Residue was placed on a flash column and eluted with 5% MeOH in CH_2Cl_2 to get a mixture of products (6.0711g, 31% yield). Rf 0.49, 0.59, 0.68 (5% MeOH in CH_2Cl_2).

EXAMPLE 76

2'-O-aminooxyethyl analogs

Various other 2'-O-aminooxyethyl analogs of nucleoside (for e.g., 2,6-diaminopurine riboside) may be prepared as compounds **154**. Thus, alkylation of 2, 6-diamino purine with (2-bromoethoxy)-*tert*-butyldimethylsilane gives 2'-O- *tert*-butyldimethylsilyloxyethyl-2,6-diaminopurine

riboside **145** and the 3'-isomer **146**. The desired 2'-O-isomer may be resolved by preparation of 5'-O-(4,4'-dimethoxytrityl)-2'-O-tert-butyldimethylsilyloxyethyl-2,6-diaminopurine riboside **147** and subjecting the mixture to column chromatography. Deprotection of the silyl group provides 5'-O-(4,4'-dimethoxytrityl)-2'-O- hydroxyethyl-2,6-diaminopurine riboside **148** which undergoes a Mitsunobu reaction to give 5'-O-(4,4'-dimethoxytrityl)- 2'-O-(2-phthalimido-N-oxyethyl)-2,6-diaminopurine riboside **149**. Treatment of **149** under Schiemann conditions effects fluorination and deprotection of the DMT group to yield 2'-O-(2-phthalimido-N-oxyethyl)-6-amino-2-fluoropurine riboside **150**. Standard protection conditions provides 5'-O-(4,4'-dimethoxytrityl)- 2'-O-(2-phthalimido-N-oxyethyl)-6-dimethyformamidine-2-fluoropurine riboside **151** and deprotection of the phthalimido function affords 5'-O-(4,4'-dimethoxytrityl)- 2'-O-aminooxyethyl-6-dimethyformamidine-2-fluoropurine riboside **152**.

Reductive amination of **152** with aldehydes or dialdehydes results in cyclic or acyclic disubstituted 2'-O-aminooxyethyl analogs **153**. Phosphitylation of **153** provides cyclic or acyclic disubstituted 2'-O-aminooxyethyl analogs **154** as the phosphoramidites.

EXAMPLE 77

2'/3'-O (2-tert-butyldimethylsilylhydroxyethyl) adenosine (155 and 156)

Adenosine (10g, 37.42mmol) was dried over P_2O_5 under high vacuum. It was then suspended in anhydrous DMF (150 mL) and NaH (1.35 g, 56.13 mmol) was added. The reaction mixture was stirred at room temperature under inert atmosphere for 30 min. Then (2-bromo ethyl)-*tert*-butyldimethylsilane (9.68mL, 4.4.90mmol) was added dropwise and the reaction mixture stirred at room temperature

overnight. DMF was removed under vacuum and to the residue dichloromethane (100mL) was added and washed with water (2 x 80mL). Dichloromethane layer was dried over anhydrous Na_2SO_4 and evaporated to dryness. Residue purified by column to get a mixture of products (4.30 g). Rf 0.49, 0.57 (10% MeOH in CH_2Cl_2)

EXAMPLE 78

2'-O-(2-methyleneiminooxyethyl) thymidine (157)

Compound **104** (3.10g, 5.48mmol) was dried over P_2O_5 under high vacuum. In a 100mL round bottom flask, triethylamine- trihydroflouride (8.93mL, 54.8mmol) was dissolved in anhydrous THF and triethylamine (3.82mL, 27.4mmol) was added. The resulting solution was immediately added to the compound **104** and the reaction mixture was stirred at room temperature overnight. Solvent was removed under vacuum. Residue obtained was placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to yield **157** as white foam (1.35 g, 75% yield). Rf 0.45 (5% MeOH in CH_2Cl_2). MS (FAB®) m/e 330 ($M+H^{\oplus}$), 352 ($M+Na^{\oplus}$).

EXAMPLE 79

5'-O-dimethoxytrityl-2'-O-(2-methyleneiminooxyethyl) thymidine (158)

Compound **157** (0.64g, 1.95mmol) was dried over P_2O_5 under high vacuum overnight. It was then co-evaporated with anhydrous pyridine (5mL). Residue dissolved in anhydrous pyridine (4.43mL) and dimethoxytrityl chloride (0.79g, 2.34mmol), and 4-dimethylaminopyridine (23.8mg, 0.2mmol) was added. Reaction mixture was stirred under inert atmosphere at ambient temperature for 4 hrs. Solvent was removed under vacuum, the residue purified by column and eluted with 5% MeOH in CH_2Cl_2 containing a few drops of pyridine to yield

158 as a foam (1.09 g, 88% yield). Rf 0.4 (5% MeOH in CH_2Cl_2). MS (Electrospray$^-$) m/e 630 (M-H$^\oplus$)

EXAMPLE 80

5'-O-dimethoxytrityl-2'-O-(2-methyleneiminooxyethyl) thymidine-3'-O-phosphoramidite (159)

Compound **158** (0.87g, 1.34mmol) was co-evaporated with toluene (10mL). Residue was then mixed with diisopropylamine tetrazolide (0.23g, 1.34mmol) and dried over P_2O_5 under high vacuum overnight. It was then flushed with argon. Anhydrous acetonitrile (6.7mL) was added to get a clear solution. To this solution 2-cyanoethyl tetraisopropylphosphorodiamidites (1.7mL, 5.36mmol) was added and the reaction mixture was stirred at room temperature for 6 hr. under inert atmosphere. Solvent was removed under vacuum, the residue was diluted with ethyl acetate (40mL), and washed with 5% $NaHCO_3$ (20mL) and brine (20mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. Residue placed on a flash column and eluted with ethyl acetate: hexane (60:40) to yield **159** (1.92 g, 80% yield). Rf 0.34 (ethyl acetate:hexane, 60:40). ^{31}P NMR ($CDCl_3$) δ 150.76 ppm, MS (Electrospray$^-$) m/e 830 (M-H$^\oplus$).

EXAMPLE 81

Attachment of nucleoside to solid support general procedure

Compound **107** (200mg, 0.31mmol) was mixed with DMAP (19mg, 16mmol), succinic anhydride (47mg, 0.47mmol), triethylamine (86mL, 0.62mmol) and dichloromethane (0.8mL) and stirred for 4 hr. The mixture was diluted with CH_2Cl_2 (50mL) and the CH_2Cl_2 layer was washed first with ice cold 10% aqueous citric acid and then with water. The organic phase was concentrated to dryness to yield **161**. Residue (**161**) was dissolved in anhydrous acetonitrile (23mL). To

this DMAP (37mg, 0.3mmol), and 2',2'-dithiobis(5-nitropyridine) (103mg, 0.33mmol) were added. The solution was stirred for 5 min. To this was added triphenylphosphine (78.69mg, 0.3mmol) in anhydrous acetonitrile (3mL). The solution was stirred for 10 min. and then CPG was added to it. The slurry was then shaken for 2 hr. It was then filtered, washed with acetonitrile and CH_2Cl_2. The functionalized CPG was dried and capped with capping solution to yield **161**. Loading capacity was determined (58.3μmol/g).

EXAMPLE 82

Synthesis of aminooxy derivatives: Alternative procedure

The diol **162** is converted to its tosylate derivative **163** by treatment with 1 equivalent of p-toluenesulfonyl chloride-pyridine followed by standard work-up. The tosylate is subsequently treated with several amino-hydroxy compounds to act as nucleophils in displacing tosylate to yield a series of oxy-amino compounds. The reaction is facilitated by preforming the anion from the amino alcohol or hydroxylamine derivative by the use of sodium hydride under anhydrous conditions.

PROCEDURE 1

Nuclease Resistance

A. Evaluation of the resistance of modified oligonucleotides to serum and cytoplasmic nucleases.

Oligonucleotides including the modified oligonucleotides of the invention can be assessed for their resistance to serum nucleases by incubation of the oligonucleotides in media containing various concentrations of fetal calf serum or adult human serum. Labeled oligonucleotides are incubated for various times, treated with protease K and then analyzed by gel electrophoresis on

20% polyacrylamide-urea denaturing gels and subsequent autoradiography. Autoradiograms are quantitated by laser densitometry. Based upon the location of the modifications and the known length of the oligonucleotide it is possible to determine the effect on nuclease degradation by the particular modification. For the cytoplasmic nucleases, a HL60 cell line is used. A post-mitochondrial supernatant is prepared by differential centrifugation and the labeled oligonucleotides are incubated in this supernatant for various times. Following the incubation, oligonucleotides are assessed for degradation as outlined above for serum nucleolytic degradation. Autoradiography results are quantitated for comparison of the unmodified and modified oligonucleotides. As a control, unsubstituted phosphodiester oligonucleotide have been found to be 50% degraded within 1 hour, and 100% degraded within 20 hours.

B. Evaluation of the resistance of modified oligonucleotides to specific endo- and exonucleases.

Evaluation of the resistance of natural and modified oligonucleotides to specific nucleases (*i.e.*, endonucleases, 3',5'-exo-, and 5',3'-exonucleases) is done to determine the exact effect of the modifications on degradation. Modified oligonucleotides are incubated in defined reaction buffers specific for various selected nucleases. Following treatment of the products with protease K, urea is added and analysis on 20% polyacrylamide gels containing urea is done. Gel products were visualized by staining using Stains All (Sigma Chemical Co.). Laser densitometry is used to quantitate the extend of degradation. The effects of the modifications are determined for specific nucleases and compared with the results obtained from the serum and cytoplasmic systems.

Nuclease resistance of oligonucleotides containing novel 2'-modifications

Series I
5'TTT TTT TTT TTT TTT*T*T*T* T 3'

SEQ ID NO 20	where T* = 5 methyl, 2'-aminooxyethoxy	2' AOE
SEQ ID NO 21	where T* = 5 methyl, 2'-dimethylaminooxyethoxy	2' DMAOE

Along with T19 diester and thioate controls, the gel purified oligos were 5' end labeled with ^{32}P, and run through the standard nuclease assay protocol. PAGE/Phosphorimaging generated images that were quantified for % Intact and % (Intact + (N-1)). The percentages were plotted to generate half-lives, which are listed in a table below. Included is the half life of the 2'-O-methoxyethyl (MOE) analog (SEQ ID NO 22) in the table. This result showed that 2'-dimethylaminooxyethyl (DMAOE) is a highly nuclease resistant modification (Fig. 14 and 15).

	2'-Modification		
	AOE	**DMAOE**	**MOE**
T1/2 of N (min)	18	60	100
T1/2 of N+(N-1) (min)	200	85% remaining at 24 hr.	300

Initial assays of the nuclease resistance of oligonucleotides capped with 2'-DMAOE modifications showed better resistance than modification 2'-O-methoxyethyl in an inter-assay comparison (Figure 13). These studies are intra-assay comparisons among several modifications in two motifs. The first motif is a full phosphodiester backbone, with a cap of 4 modified nucleotides beginning at the 3'-most nucleotide. The second motif is similar, but contains a

single phosphorothioate at the 3'-most inter nucleotide linkage.

Series II
5' TTT TTT TTT TTT TTT T*T*T* T* 3'

SEQ ID NO 23	where T* = 5 methyl, 2'-dimethylaminooxyethyl
SEQ ID NO 24	where T* = 5 methyl, 2'-O-methoxyethyl
SEQ ID NO 25	where T* = 5 methyl, 2'-O-propyl

Series III
5' TTT TTT TTT TTT TTT TTT*T 3'

SEQ ID NO 26	where T* = 5 methyl, 2'-dimethylaminooxyethyl
SEQ ID NO 27	where T* = 5 methyl, 2'-O-methoxyethyl

Along with a T19 phosphorothioate control, the oligos were gel purified and run through the standard nuclease protocol. From these assays SEQ ID NO: 23 proved to be the next most resistant oligonucleotide. SEQ ID NO: 24 is degraded more readily, and SEQ ID NO:25 is degraded rather quickly. The gel shows some reaction products at the bottom of the gel, but little n-2 and n-3 of the resistant oligonucleotides. These products appear to be the result of endonucleolytic cleavage by SVPD. This type of activity is always present at a basal rate, but is not usually seen due to the overwhelming predominance of 3' exonuclease activity on most oligonucleotides. However, these oligonucleotides are so extraordinarily resistant to 3' exonucleases that the endonuclease activity is responsible for a majority of the cleavage events on the full-length oligo. 2'-deoxy phosphodiester products of the endonuclease reactions are then rapidly cleaved to monomers. Two sets of quantitation are done for these reactions. One counts only 3'-exonuclease products, and the other counts products for all reactions. In either case, the half-life of a SEQ ID NO: 23 is longer

than 24 hours. For SEQ ID NO: 24 the half life of the exonuclease activity is over 24 hours while the other type of Quantitation gives a half-life of about 100 min. The oligonucleotides of the motif containing a single phosphorothioate linkage (SEQ ID NO: 26 and SEQ ID NO: 27) are substrates for the endonuclease activity described above, but no products of 3′ exonuclease activity are detected in the time course of this assay.

Table 2
Oligonucleotides synthesized with 2′-dimethylaminooxyethyl thymidine (T-2′-DMAOE)

SEQ ID NO:	Sequence	Mass	
		Exp.	Obs.
28	5′- CTCGTACCT*TTCCGGTCC-3′	5784.20	5784.09
29	5′-T*CCAGGT*GT*CCGCAT*C-3′	5548.74	5549.05
30	5′-GCGT*T*T*T*T*T*T*T*T*T*GCG-3′	6208.74	6210.52
31	5′-TTTTTTTTTTTTTTT*T*T*T*T-3′	6433.45	6433.79
32	5′-T*T*T*T*-3′	1869.96	1869.5
33	5′-TTTTTTTTTTTTTTTT*T*T*$_S$T*-3′	6449.45	6449.15
34	TTTTTTTTTTTTTTTT*T*T*T*-3′	6433.51	6433.19
35	5′-T*T*-3′	648.49	648.4

Table 3
Oligonucleotides synthesized with 2′-dimethylaminooxyethyl adenosine (A-2′-DMAOE)

	SEQ ID NO:	Sequence	Mass	
			Exp.	Obs.
1	36	5′- CTCGTACCA*TTCCGGTCC-3′	5490.21	5490.86
2	37	5′-GGA*CCGGA*A*GGTA*CGA*G-3′	5824.96	5826.61
3	38	5′-A*CCGA*GGA*GGA*TCA*TGTCGTA*CGC-3′	6947.9	6947.28

Table 4
Oligonucleotides synthesized with 2'-O-methyleneiminooxyethyl adenosine

	SEQ ID NO:	Sequence	Mass	
			Ex.	Obs.
	39	5'-CTCGTACCA*TTCCGGTCC-3'	5470.20	5472.50
	40	5'-A*CCGA*GGA*TCA*TGTCGTA*CGC-3'	6866.42	6865.88
	41	5'-GGA*CCGGA*A*GGTA*CGA*G-3'	5743.12	5743.82

Table 5
Oligonucleotides synthesized with 2'-O-mehtyleneiminooxyethyl thymidine

	SEQ ID NO:	Sequence	Mass	
			Exp.	Obs.
1	42	5'-CTCGTACCT*TTCCGGTCC-3'	5466.21	5462.25
2	43	5'-T*CCAGGT*GT*CCGCAT*C-3'	5179.44	5178.96
3	44	5'-TTTTTTTTTTTTTTT*T*T*T*T-3'	6369.45	6367.79

Table 6
Tm advantage of 2'-DMAOE modification over 2'-deoxy phosphodiesters and phosphorothioates

	SEQ. ID NO:	SEQUENCE	Tm	ΔTm/mod against RNA compared to unmodified DNA	ΔTm/mod against RNA compared to unmodified deoxy-phosphoro-thioate
	45	5'-CTCGTAC-CT*T-TCCGGTCC-3'	65.44	0.24	1.04
	46	5'-T*CCAGGT*GT*C-CGCAT*C-3'	67.90	1.12	2.20
	47	5'-GCGT*T*T*T*T*T* T*T*T*T*GCG-3'	62.90	1.46	2.36

ΔTm is based on reported literature values for DNA and phosphorothioate oligonucleotides.

PROCEDURE 2

Ras-Luciferase Reporter Gene Assembly

The ras-luciferase reporter genes described in this study are assembled using PCR technology. Oligonucleotide primers are synthesized for use as primers for PCR cloning of the 5'-regions of exon 1 of both the mutant (codon 12) and non-mutant (wild-type) human H-ras genes. H-ras gene templates are purchased from the American Type Culture Collection (ATCC numbers 41000 and 41001) in Bethesda, MD. The oligonucleotide PCR primers 5'-ACA-TTA-TGC-TAG-CTT-TTT-GAG-TAA-ACT-TGT-GGG-GCA-GGA-GAC-CCT-GT-3' (sense) (SEQ ID NO:16), and 5'-GAG-ATC-TGA-AGC-TTC-TGG-ATG-GTC-AGC-GC-3' (antisense) (SEQ ID NO:17), are used in standard PCR reactions using mutant and non-mutant H-ras genes as templates. These primers are expected to produce a DNA product of 145 base pairs corresponding to sequences -53 to +65 (relative to the translational initiation site) of normal and mutant H-ras, flanked by NheI and HindIII restriction endonuclease sites. The PCR product is gel purified, precipitated, washed and resuspended in water using standard procedures.

PCR primers for the cloning of the *P. pyralis* (firefly) luciferase gene were designed such that the PCR product would code for the full-length luciferase protein with the exception of the amino-terminal methionine residue, which would be replaced with two amino acids, an amino-terminal lysine residue followed by a leucine residue. The oligonucleotide PCR primers used for the cloning of the luciferase gene are 5'-GAG-ATC-TGA-AGC-TTG-AAG-ACG-CCA-AAA-ACA-TAA-AG-3' (sense) (SEQ ID NO:18), and 5'-ACG-CAT-CTG-GCG-CGC-CGA-TAC-CGT-CGA-CCT-CGA-3' (antisense) (SEQ ID NO:19), are used in standard PCR reactions using a commercially available plasmid (pT3/T7-Luc) (Clontech), containing the luciferase reporter gene, as a template. These primers are expected to yield a product of approximately 1.9 kb corresponding to the luciferase gene, flanked by HindIII and BssHII

restriction endonuclease sites. This fragment is gel purified, precipitated, washed and resuspended in water using standard procedures.

To complete the assembly of the ras-luciferase fusion reporter gene, the ras and luciferase PCR products are digested with the appropriate restriction endonucleases and cloned by three-part ligation into an expression vector containing the steroid-inducible mouse mammary tumor virus promotor MMTV using the restriction endonucleases NheI, HindIII and BssHII. The resulting clone results in the insertion of H-ras 5' sequences (-53 to +65) fused in frame with the firefly luciferase gene. The resulting expression vector encodes a ras-luciferase fusion product which is expressed under control of the steroid-inducible MMTV promoter.

PROCEDURE 3

Transfection of Cells with Plasmid DNA

Transfections are performed as described by Greenberg in *Current Protocols in Molecular Biology*, Ausubel *et al.*, Eds., John Wiley and Sons, New York, with the following modifications: HeLa cells are plated on 60 mm dishes at 5 x 10^5 cells/dish. A total of 10 μg of DNA is added to each dish, of which 9 μg is ras-luciferase reporter plasmid and 1 μg is a vector expressing the rat glucocorticoid receptor under control of the constitutive Rous sarcoma virus (RSV) promoter. Calcium phosphate-DNA coprecipitates are removed after 16-20 hours by washing with Tris-buffered saline (50 Mm Tris-Cl (pH 7.5), 150 mM NaCl) containing 3 mM EGTA. Fresh medium supplemented with 10% fetal bovine serum is then added to the cells. At this time, cells are pre-treated with antisense oligonucleotides prior to activation of reporter gene expression by dexamethasone.

PROCEDURE 4

Oligonucleotide Treatment of Cells

Immediately following plasmid transfection, cells are thrice washed with OptiMEM (GIBCO), and prewarmed to 37°C. 2 ml of OptiMEM containing 10 μg/ml N-[1-(2,3-diolethyloxy)propyl]-N,N,N,-trimethylammonium chloride (DOTMA) (Bethesda Research Labs, Gaithersburg, MD) is added to each dish and oligonucleotides are added directly and incubated for 4 hours at 37°C. OptiMEM is then removed and replaced with the appropriate cell growth medium containing oligonucleotide. At this time, reporter gene expression is activated by treatment of cells with dexamethasone to a final concentration of 0.2 μM. Cells are harvested 12-16 hours following steroid treatment.

PROCEDURE 5

Luciferase Assays

Luciferase is extracted from cells by lysis with the detergent Triton X-100, as described by Greenberg in *Current Protocols in Molecular Biology*, Ausubel *et al.*, Eds., John Wiley and Sons, New York. A Dynatech ML1000 luminometer is used to measure peak luminescence upon addition of luciferin (Sigma) to 625 μM. For each extract, luciferase assays are performed multiple times, using differing amounts of extract to ensure that the data are gathered in the linear range of the assay.

PROCEDURE 6

Antisense Oligonucleotide Inhibition of ras-Luciferase Gene Expression

A series of antisense phosphorothioate oligonucleotide analogs targeted to the codon-12 point mutation of activated H-ras are tested using the ras-luciferase reporter gene system described in the foregoing examples. This series comprised a basic sequence and analogs of that basic sequence. The basic sequence is of known activity as reported in International Publication

Number WO 92/22651 identified above. In both the basic sequence and its analogs, each of the nucleotide subunits incorporated phosphorothioate linkages to provide nuclease resistance. Each of the analogs incorporated nucleotide subunits that contained 2'-O-substitutions and 2'-deoxy-*erythro*-pentofuranosyl sugars. In the analogs, a subsequence of the 2'-deoxy-*erythro*-pentofuranosyl sugar-containing subunits is flanked on both ends by subsequences of 2'-O-substituted subunits. The analogs differed from one another with respect to the length of the subsequence of the 2'-deoxy-*erythro*-pentofuranosyl sugar containing nucleotides. The length of these subsequences are varied by 2 nucleotides between 1 and 9 total nucleotides. The 2'-deoxy-*erythro*-pentofuranosyl nucleotide sub-sequences are centered at the point mutation of the codon-12 point mutation of the activated ras.

PROCEDURE 7

Diagnostic Assay for the Detection of mRNA overexpression

Oligonucleotides are radiolabeled after synthesis by ^{32}P labeling at the 5' end with polynucleotide kinase. Sambrook et al. (*"Molecular Cloning. A Laboratory Manual,"* Cold Spring Harbor Laboratory Press, 1989, Volume 2, pg. 11.31-11.32). Radiolabeled oligonucleotide is contacted with tissue or cell samples suspected of mRNA overexpression, such as a sample from a patient, under conditions in which specific hybridization can occur, and the sample is washed to remove unbound oligonucleotide. A similar control is maintained wherein the radiolabeled oligonucleotide is contacted with normal cell or tissue sample under conditions that allow specific hybridization, and the sample is washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide and is quantitated using a scintillation counter or other routine means. Comparison of the radioactivity remaining in the samples from normal

and diseased cells indicates overexpression of the mRNA of interest.

Radiolabeled oligonucleotides of the invention are also useful in autoradiography. Tissue sections are treated with radiolabeled oligonucleotide and washed as described above, then exposed to photographic emulsion according to standard autoradiography procedures. A control with normal cell or tissue sample is also maintained. The emulsion, when developed, yields an image of silver grains over the regions overexpressing the mRNA, which is quantitated. The extent of mRNA overexpression is determined by comparison of the silver grains observed with normal and diseased cells.

Analogous assays for fluorescent detection of mRNA expression use oligonucleotides of the invention which are labeled with fluorescein or other fluorescent tags. Labeled DNA oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. ß-cyanoethyldiisopropyl phosphoramidites are purchased from Applied Biosystems (Foster City, CA). Fluorescein-labeled amidites are purchased from Glen Research (Sterling, VA). Incubation of oligonucleotide and biological sample is carried out as described for radiolabeled oligonucleotides except that instead of a scintillation counter, a fluorescence microscope is used to detect the fluorescence. Comparison of the fluorescence observed in samples from normal and diseased cells enables detection of mRNA overexpression.

PROCEDURE 8

Detection of Abnormal mRNA Expression

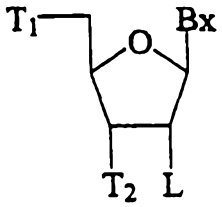
Tissue or cell samples suspected of expressing abnormal mRNA are incubated with a first ^{32}P or fluorescein-labeled oligonucleotide which is targeted to the wild-type (normal) mRNA. An identical sample of cells or tissues is incubated with a second labeled

oligonucleotide which is targeted to the abnormal mRNA, under conditions in which specific hybridization can occur, and the sample is washed to remove unbound oligonucleotide. Label remaining in the sample indicates bound oligonucleotide and can be quantitated using a scintillation counter, fluorimeter, or other routine means. The presence of abnormal mRNA is indicated if binding is observed in the case of the second but not the first sample.

Double labeling can also be used with the oligonucleotides and methods of the invention to specifically detect expression of abnormal mRNA. A single tissue sample is incubated with a first ^{32}P-labeled oligonucleotide which is targeted to wild-type mRNA, and a second fluorescein-labeled oligonucleotide which is targeted to the abnormal mRNA, under conditions in which specific hybridization can occur. The sample is washed to remove unbound oligonucleotide and the labels are detected by scintillation counting and fluorimetry. The presence of abnormal mRNA is indicated if the sample does not bind the ^{32}P-labeled oligonucleotide (*i.e.*, is not radioactive) but does retain the fluorescent label (*i.e.*, is fluorescent).

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A compound having the structure:

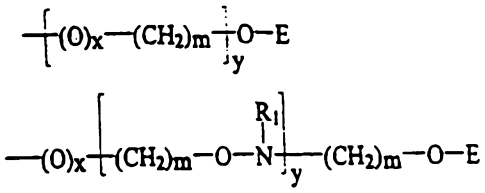


wherein:

B_X is purine or pyrimidine heterocyclic base;

T_1 and T_2, independently, are OH, a hydroxyl protecting group, an activated phosphate group, a nucleotide, a nucleoside, or an oligonucleotide;

L has one of the structures:



where

m is from 1 to 10;

y is from 1 to 10;

x is 1;

E is $N(R_1)(R_2)$ or $N=C(R_1)(R_2)$; and

each R_1 and R_2 is, independently, H, C_1-C_{10} alkyl, a nitrogen protecting group, or R_1 and R_2, together, are a nitrogen protecting group or wherein R_1 and R_2 are joined in a ring structure that can include at least one heteroatom selected from N and O.

2. The compound of claim 1 wherein Bx is adenine, guanine, hypoxanthine, uracil, thymine, cytosine, 2-aminoadenine or 5-methylcytosine.

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3. The compound of claim 1 wherein R_1 and R_2 are independently H or C_1-C_{10} alkyl.

4. The compound of claim 1 wherein R_1 and R_2 are joined in a ring structure that can include at least one heteroatom selected from N and O.

5. The compound of claim 4 wherein said ring structure is imidazole, piperidine, morpholine or a substituted piperazine.

6. The compound of claim 5 wherein said substituted piperazine is substituted with a C_1-C_{12} alkyl.

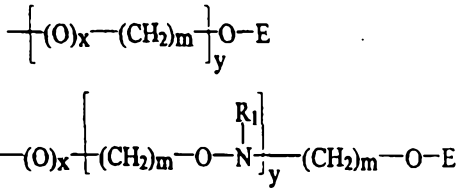
7. The compound of claim 1 wherein both of said T_1 and T_2 are oligonucleotides or one of said T_1 and T_2 is an oligonucleotide and the other is a hydroxyl protecting group.

8. A compound of the structure:

Q-L

wherein

L has one of the structures:



where

m is from 0 to 10;

y is from 1 to 10;

E is $N(R_1)(R_2)$ or $N=C(R_1)(R_2)$;

each R_1 and R_2 is, independently, H, C_1-C_{10} alkyl, a nitrogen protecting group, or R_1 and R_2, together, are a nitrogen protecting group or wherein R_1 and R_2 are joined in a ring structure

that can include at least one heteroatom selected from N and O;

and X and Q are selected such that:

when X is 1, then Q is:

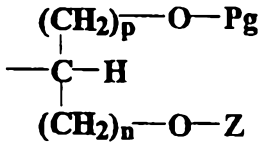
a nucleoside substituted with said L group at a 2', 3' or 5' position; or

a 3'-phosphitylated nucleoside substituted with said L group at a 2' or 5' position; or

a 2'-phosphitylated nucleoside substituted with said L group at a 3' or 5' position; or

an oligonucleotide that includes a nucleotide substituted at a 2', 3' or 5' position with said L group; or

when X is 0, then Q is a compound of the structure:



where

n and p are, independently, from 0 to 10 with the sum of n and p being greater than 2 and less then 11;

Pg is a hydroxyl protecting group; and

Z is a solid suport or a protected and activated phosphorus moiety.

9. A compound of claim 8 wherein Q is:

a nucleoside substituted with said L group at a 2', 3' or 5' position;

a 3'-phosphitylated nucleoside substituted with said L group at a 2' or 5' position;

a 2'-phosphitylated nucleoside substituted with said L group at a 3' or 5' position; or

an oligonucleotide that includes a nucleotide substituted at a 2', 3' or 5' position with said L group.

10. A compound of claim 8 where Q is a nucleoside substituted with said L group at a 2', 3' or 5' position.

11. A compound of claim 10 where Q is a nucleoside substituted with said L group at its 2' position.

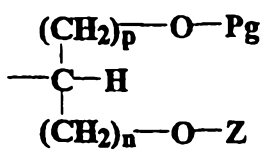
12. A compound of claim 10 where Q is 3'-phosphitylated nucleoside substituted with said L group at its 2' position.

13. A compound of claim 9 where Q is an oligonucleotide that includes at least one nucleotide substituted at a 2', 3' or 5' position with said L group.

14. A compound of claim 13 where Q is an oligonucleotide that includes at least one nucleotide substituted at its 2'position with said L group.

15. A compound of claim 9 where Q is an oligonucleotide that includes at least one nucleotide substituted at its 2'positions with a aminooxyethyl group.

16. A compound of claim 8 where Q is a compound of the structure:



17. A compound of claim 10 where Z is a solid support.

18. A compound of claim 17 where Z is a protected and activated phosphorous moiety.

19. A compound of claim 18 wherein Z is a cyanoethoxy-N,N-diisopropyl phosphoramidite group.

20. A compound of claim 16 where Pg is dimethoxytrityl.

21. A compound of claim 16 where p is 1 and n is 4.

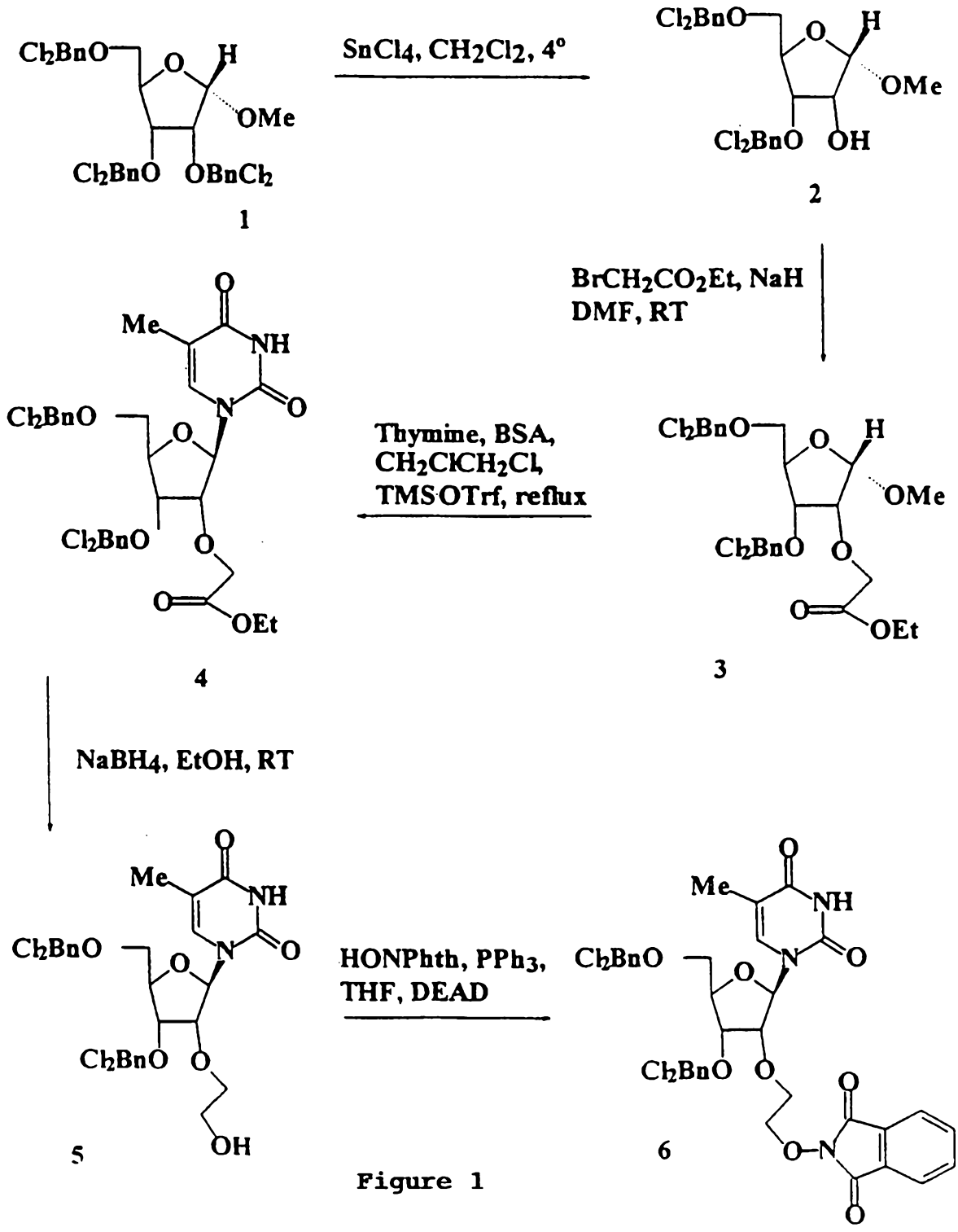

Cl2BnO
OMe
Cl2BnO OBnCl2
1
SnCl4, CH2Cl2, 4°
Cl2BnO
OMe
Cl2BnO OH
2
BrCH2CO2Et, NaH
DMF, RT
Me
NH
Cl2BnO
Cl2BnO
OEt
4
Thymine, BSA,
CH2ClCH2Cl,
TMSOTrf, reflux
Cl2BnO
OMe
Cl2BnO
OEt
3
NaBH4, EtOH, RT
Me
NH
Cl2BnO
Cl2BnO
OH
5
HONPhth, PPh3,
THF, DEAD
Me
NH
Cl2BnO
Cl2BnO
6

Figure 1

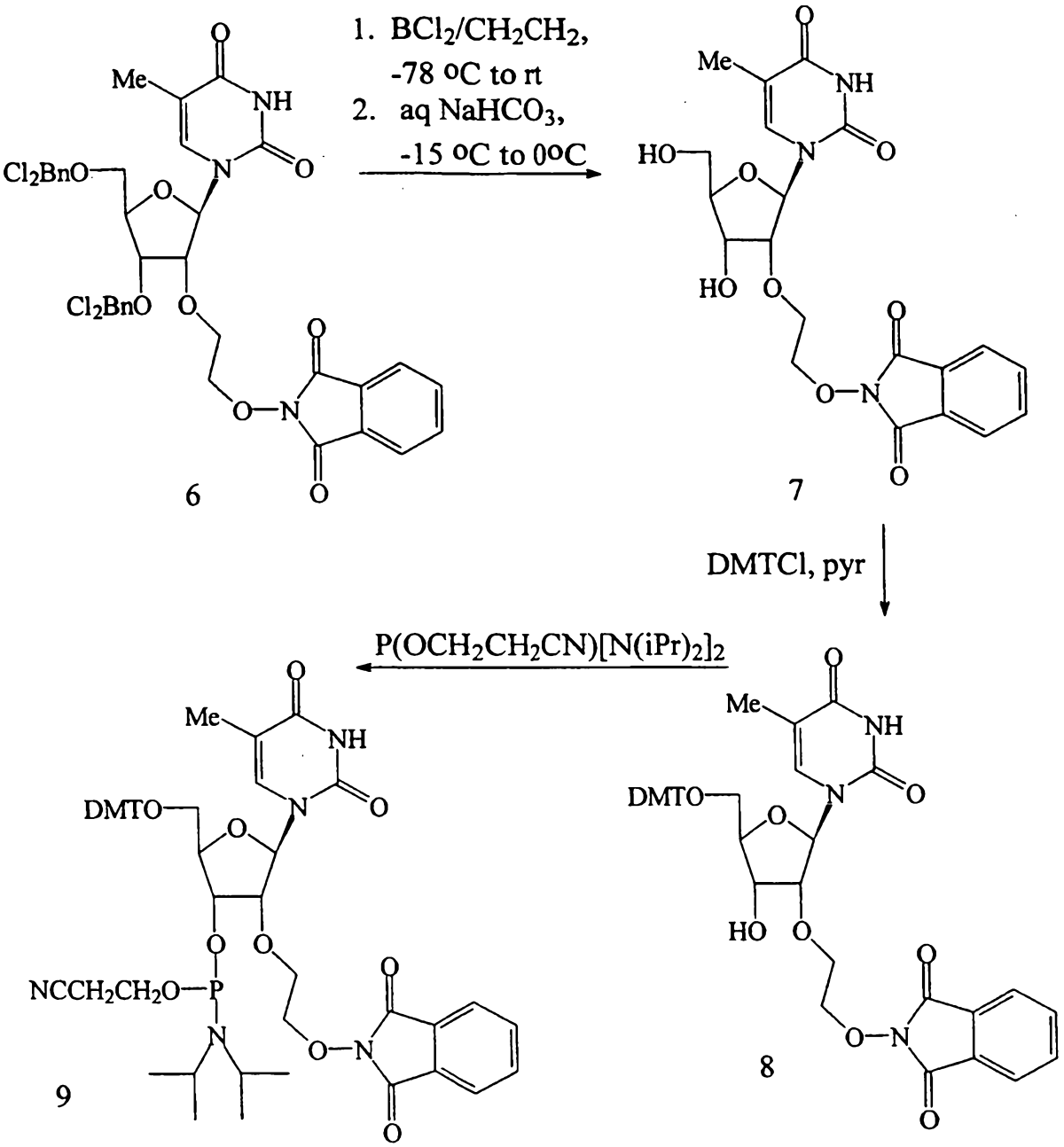

1. BCl_2/CH_2CH_2,
-78 °C to rt
2. aq $NaHCO_3$,
-15 °C to 0°C
Cl_2BnO
Cl_2BnO
Me
NH
HO
HO
DMTCl, pyr
$P(OCH_2CH_2CN)[N(iPr)_2]_2$
DMTO
$NCCH_2CH_2O$—P
6
7
8
9

Figure 2

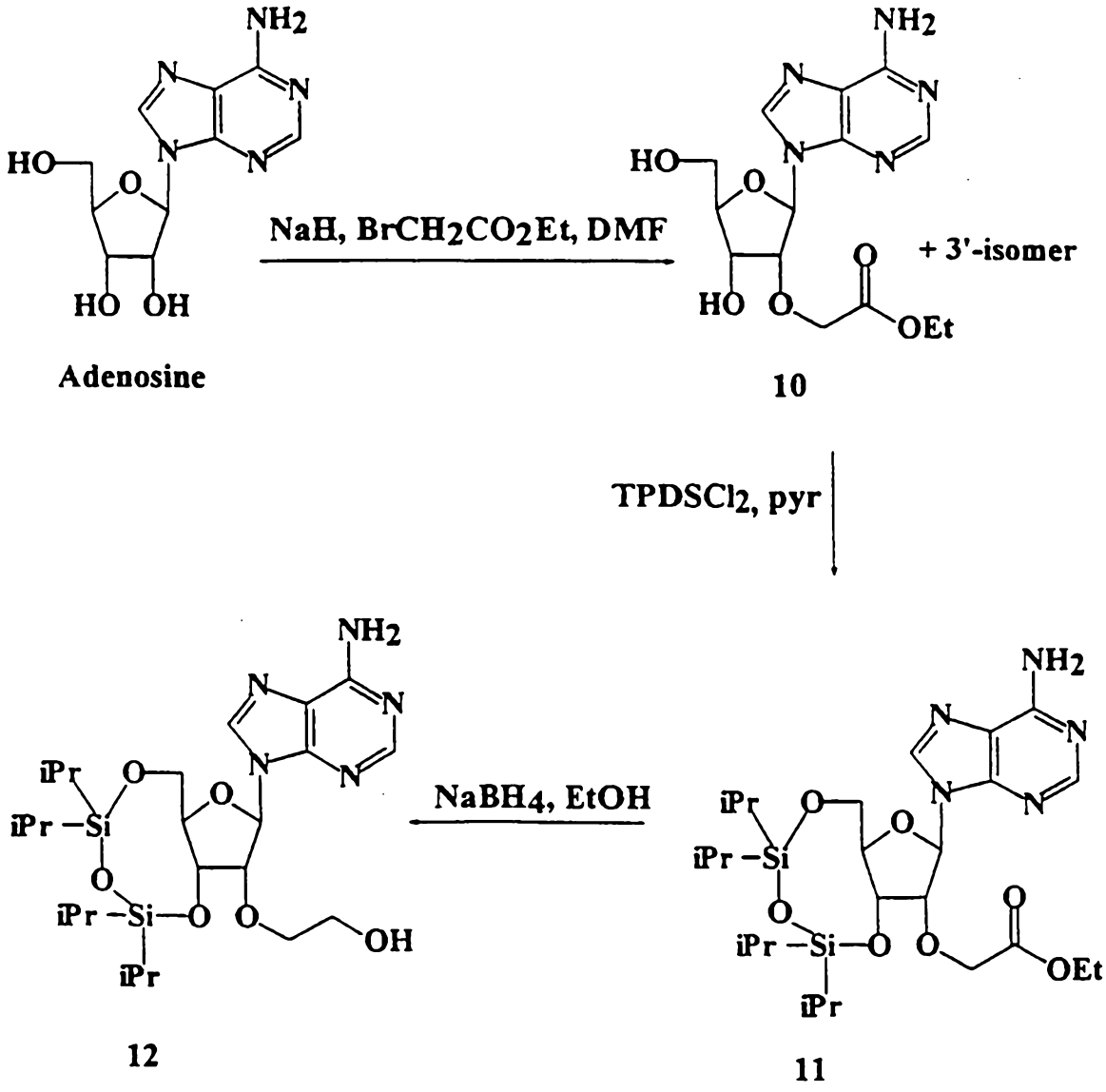

NaH, BrCH2CO2Et, DMF
+ 3'-isomer
Adenosine
10
TPDSCl2, pyr
NaBH4, EtOH
12
11

Figure 3

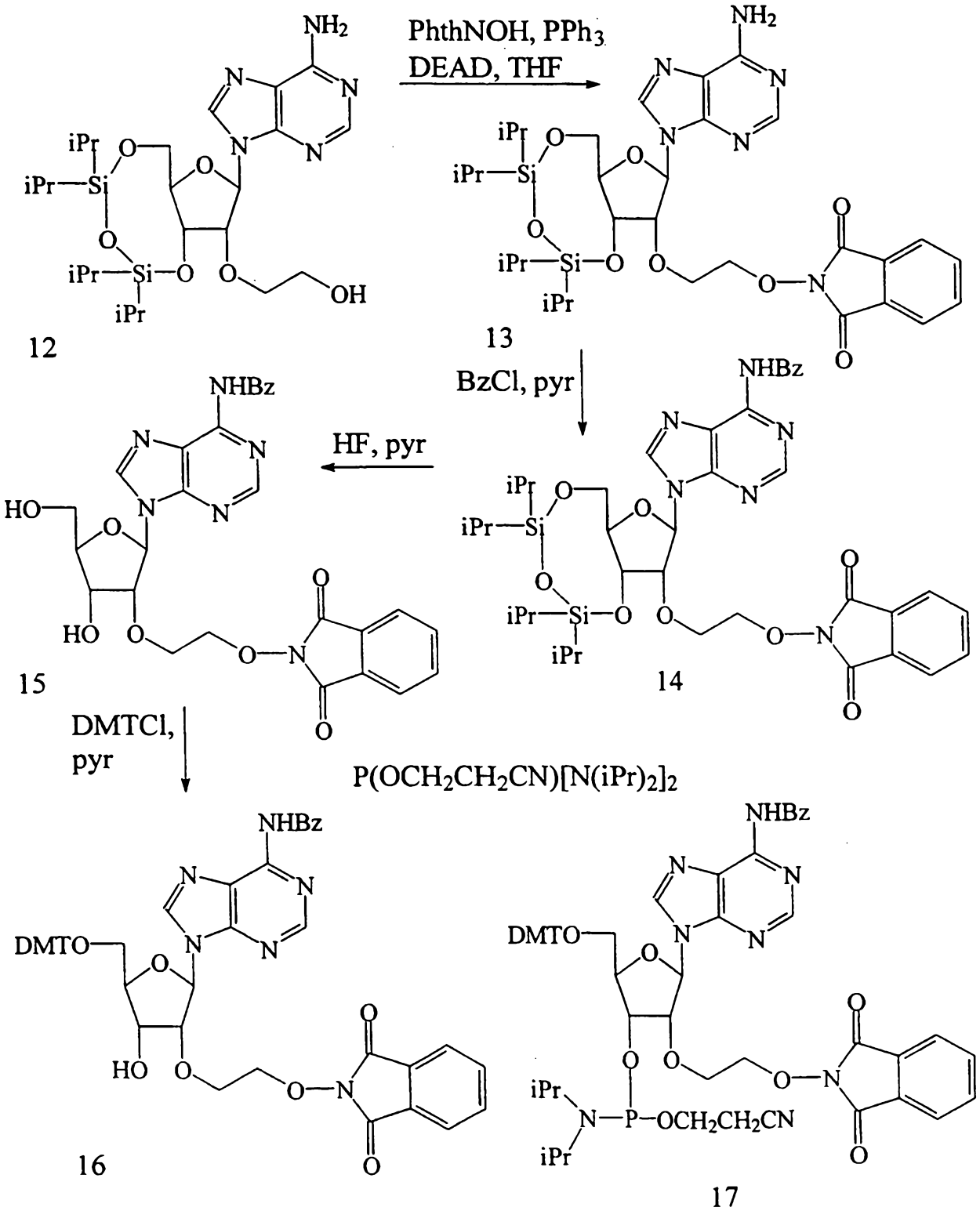

PhthNOH, PPh3
DEAD, THF
12
13
BzCl, pyr
HF, pyr
15
14
DMTCl,
pyr
P(OCH2CH2CN)[N(iPr)2]2
16
17

FIGURE 4

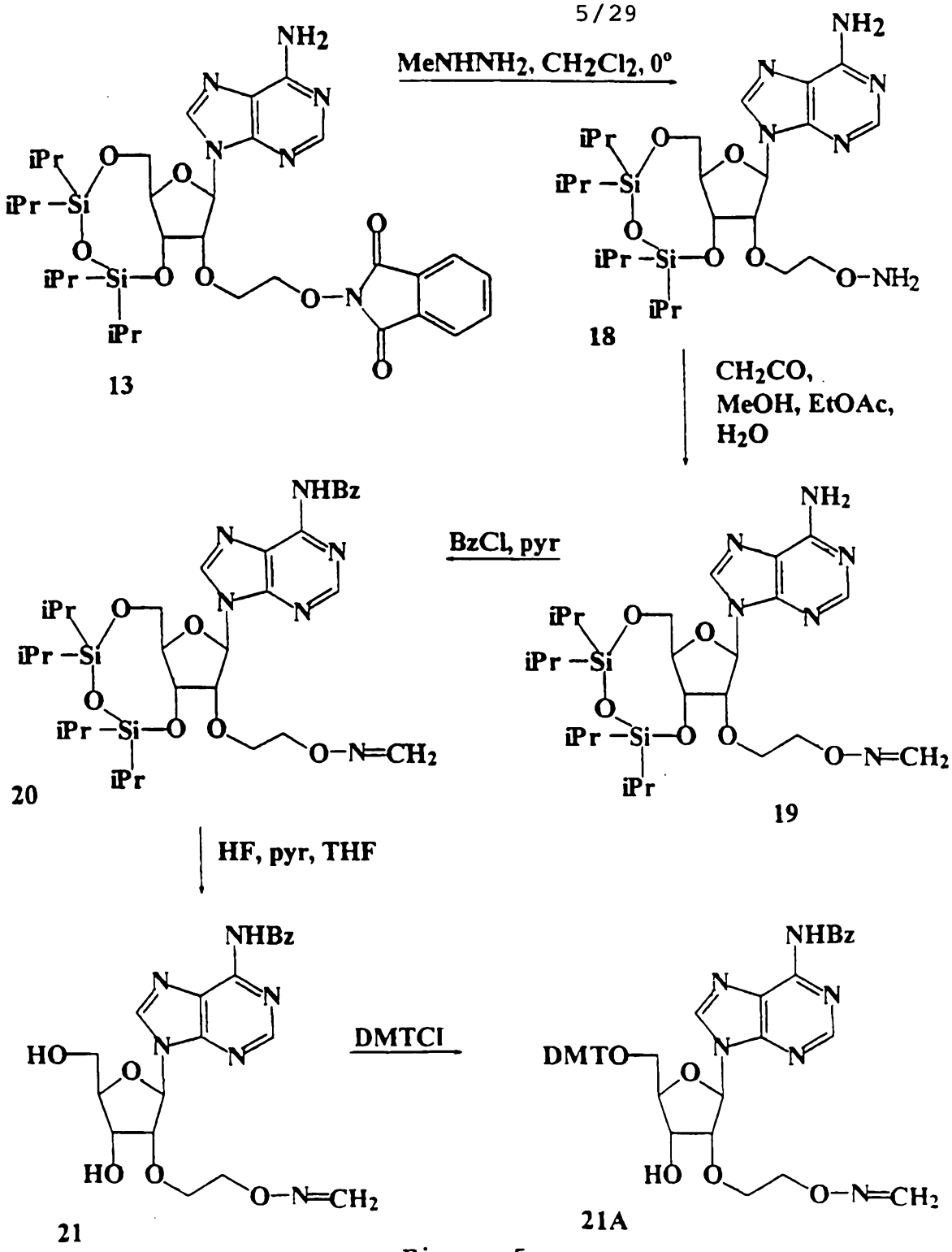

MeNHNH2, CH2Cl2, 0°
CH2CO, MeOH, EtOAc, H2O
BzCl, pyr
HF, pyr, THF
DMTCl
13
18
20
19
21
21A

Figure 5

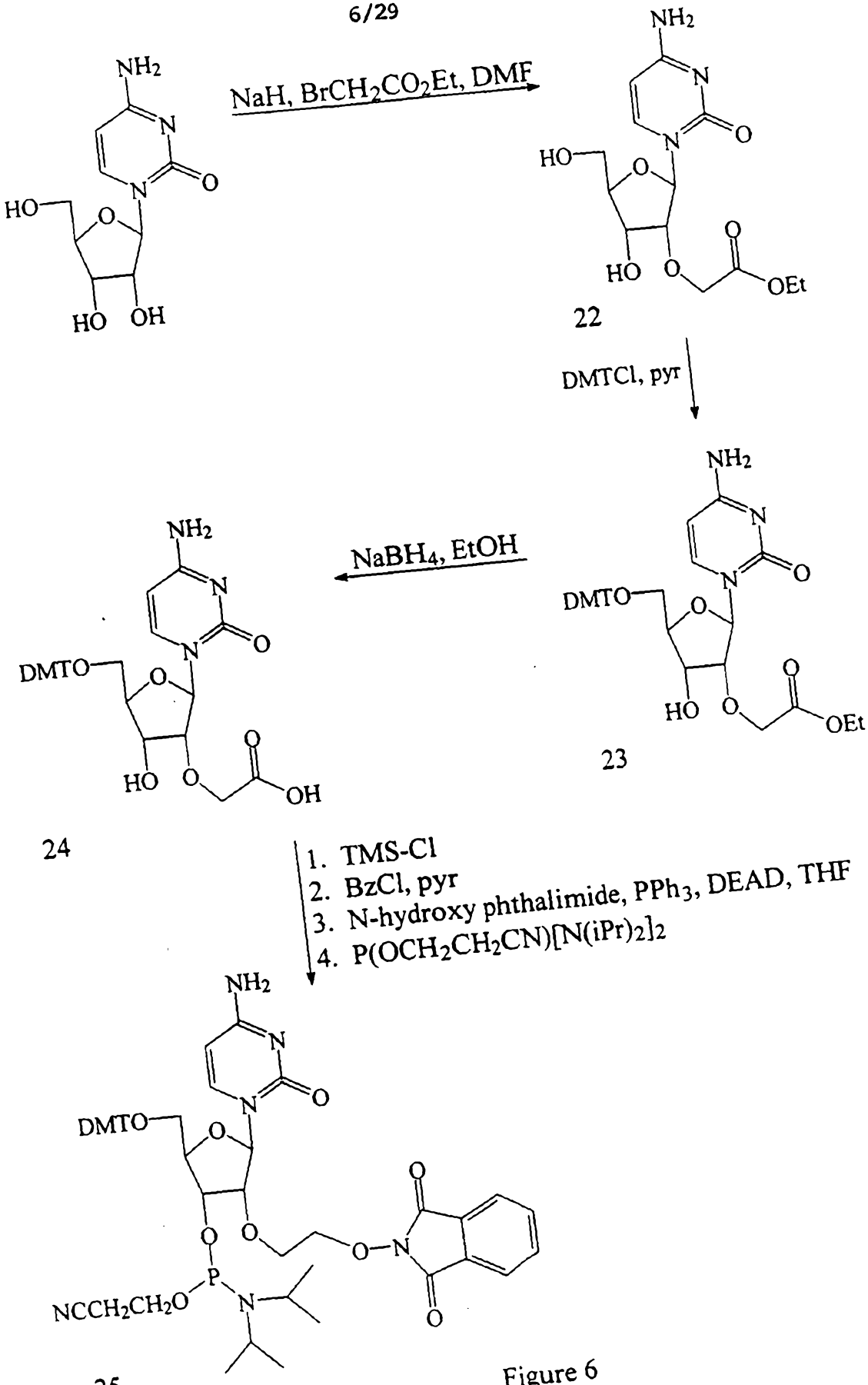

NaH, BrCH2CO2Et, DMF
22
DMTCl, pyr
23
NaBH4, EtOH
24
1. TMS-Cl
2. BzCl, pyr
3. N-hydroxy phthalimide, PPh3, DEAD, THF
4. P(OCH2CH2CN)[N(iPr)2]2
25

Figure 6

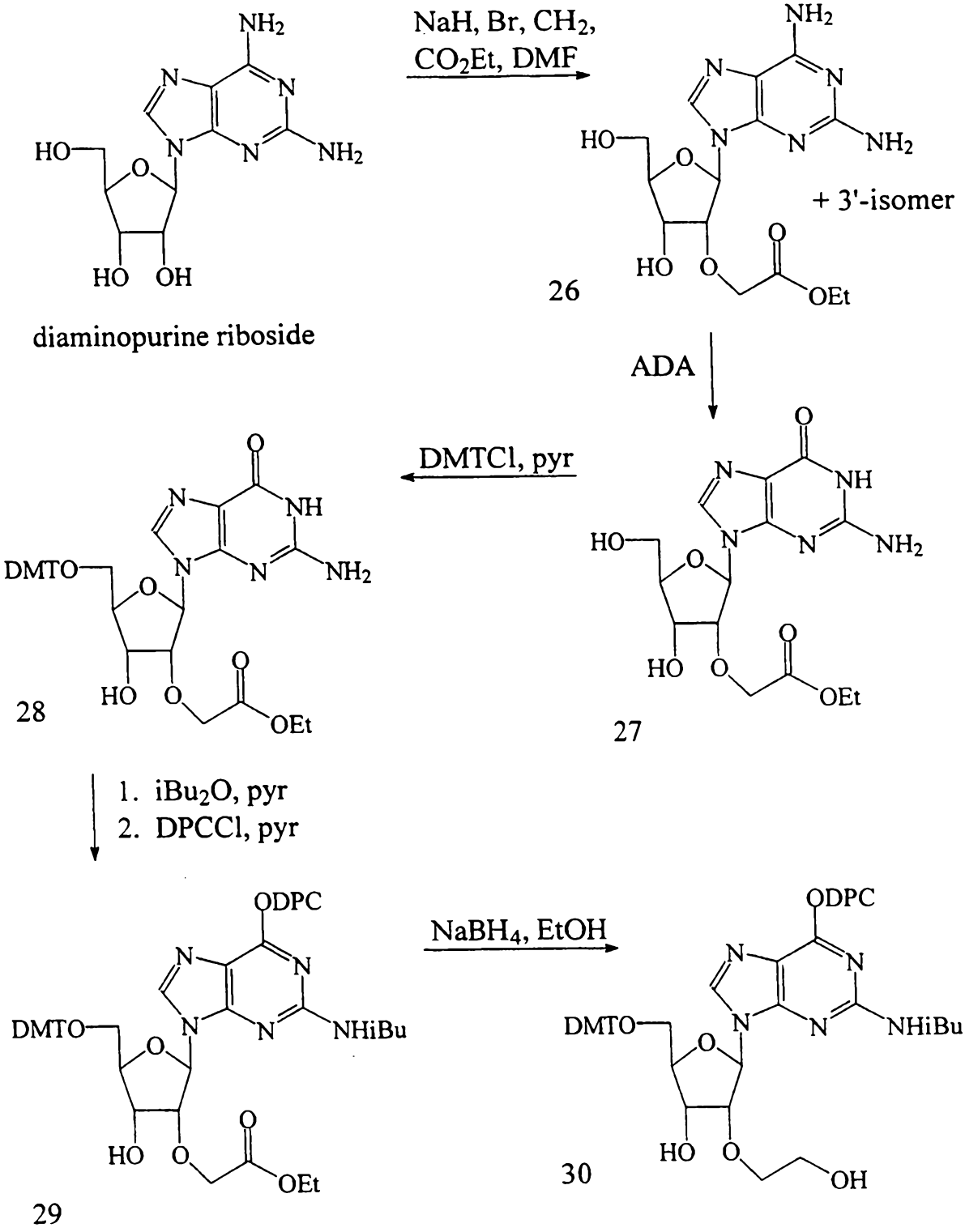
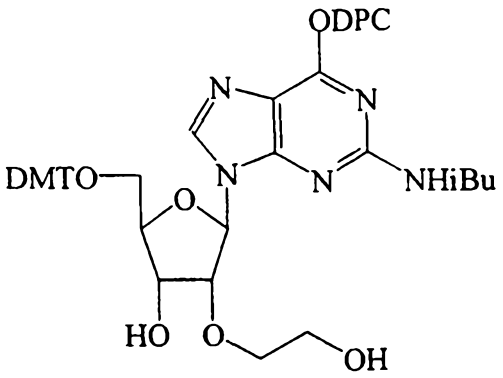
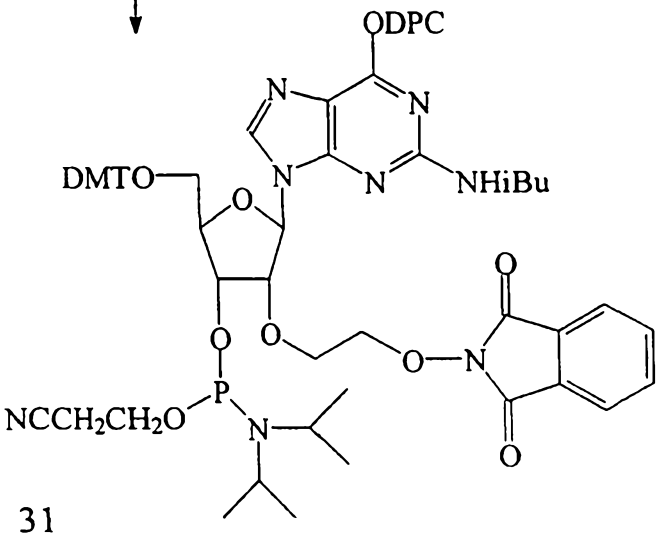

NaH, Br, CH2,
CO2Et, DMF
+ 3'-isomer
26
diaminopurine riboside
ADA
DMTCl, pyr
28
27
1. iBu2O, pyr
2. DPCCl, pyr
NaBH4, EtOH
29
30

Figure 7



30

1. N-hydroxy phthalimide, PPh_3, DEAD, THF
2. $P(OCH_2CH_2CN)[N(iPr)_2]_2$



31

Figure 8

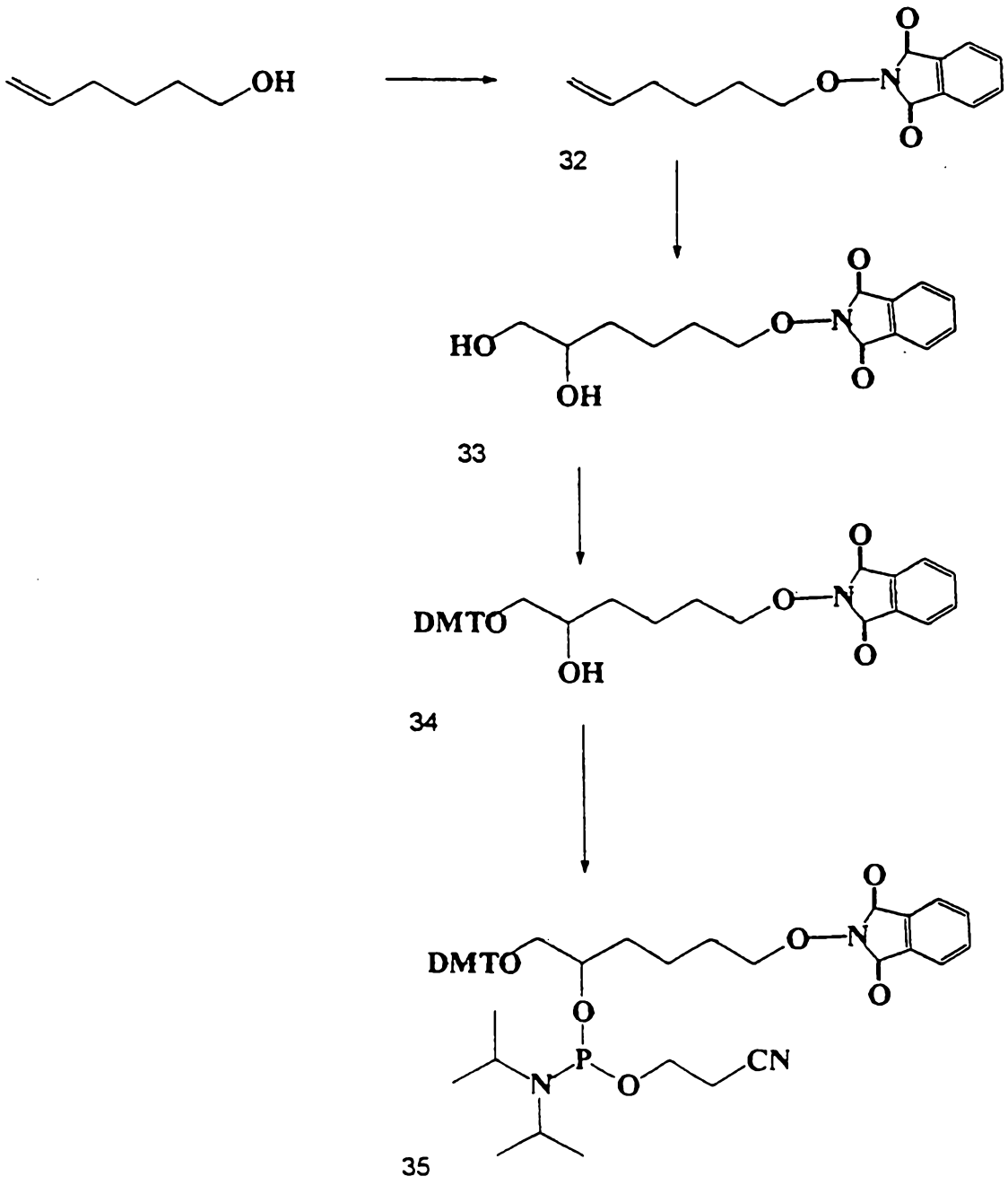


Figure 9

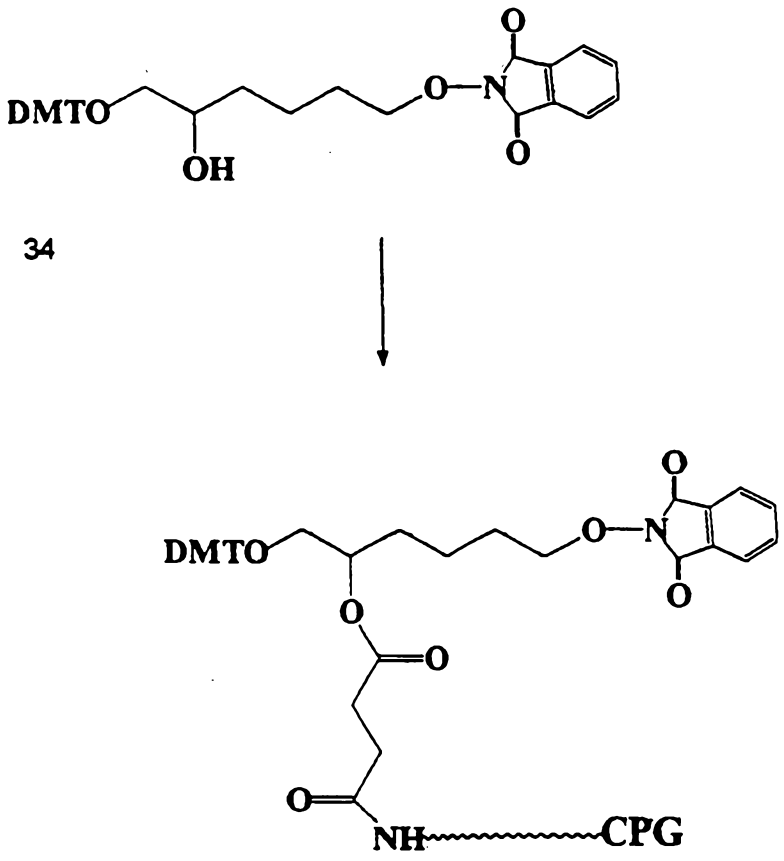


Figure 10

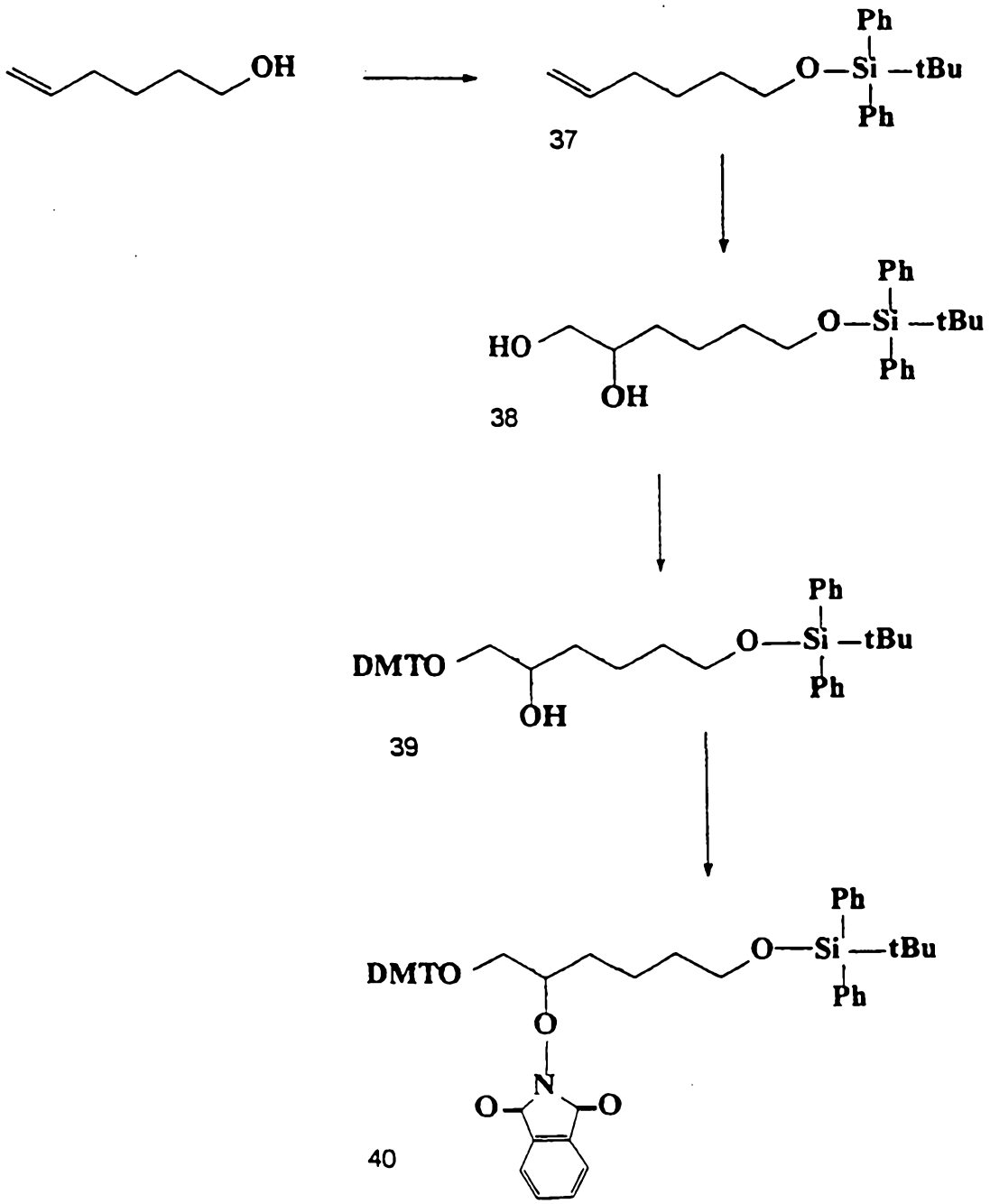


Figure 11

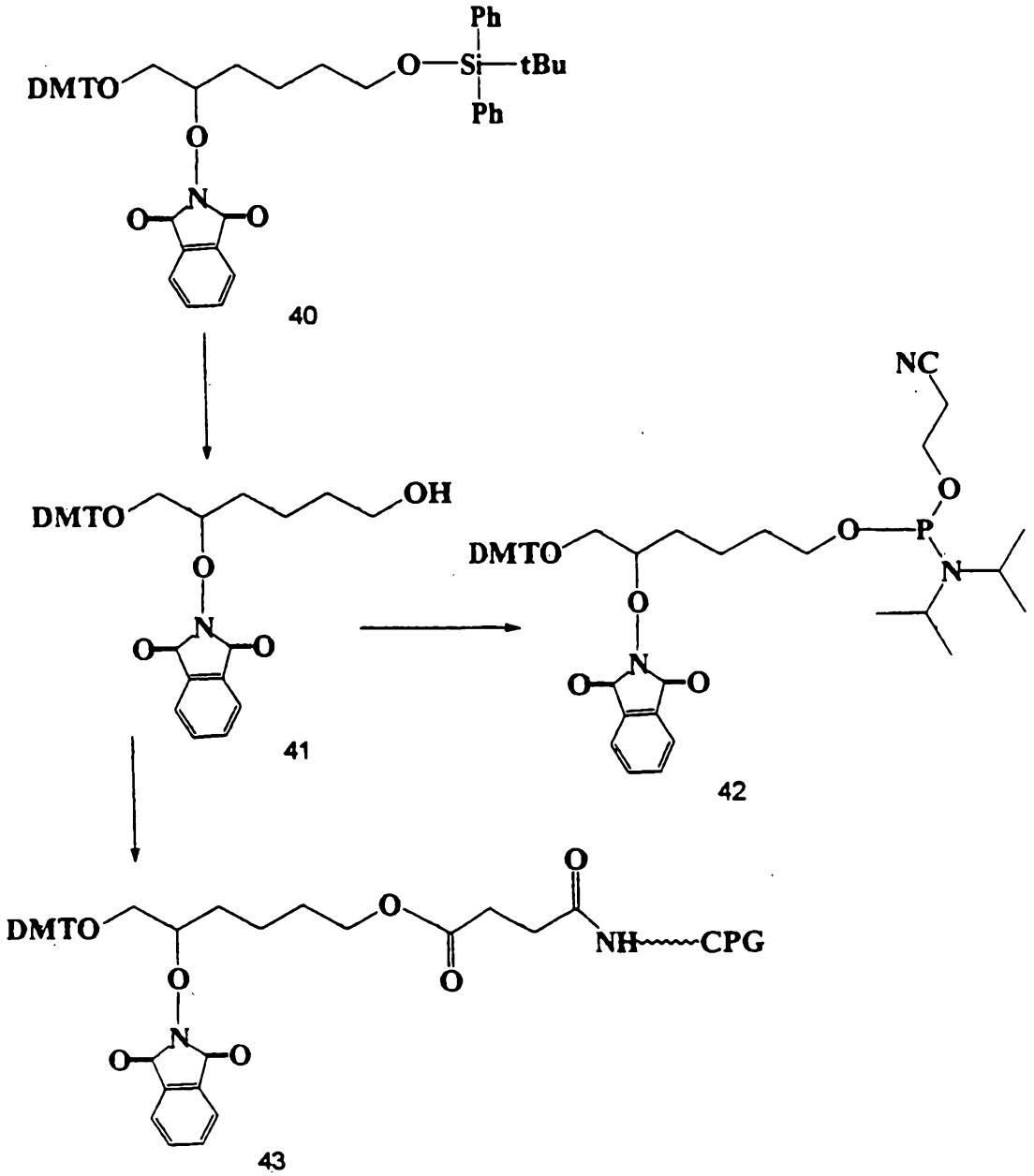

Ph
DMTO
O
Si
tBu
Ph
O
N
O
O
40
OH
DMTO
O
N
O
O
41
NC
O
P
DMTO
O
N
N
O
O
42
O
O
DMTO
O
NH
CPG
O
O
N
O
O
43

Figure 12

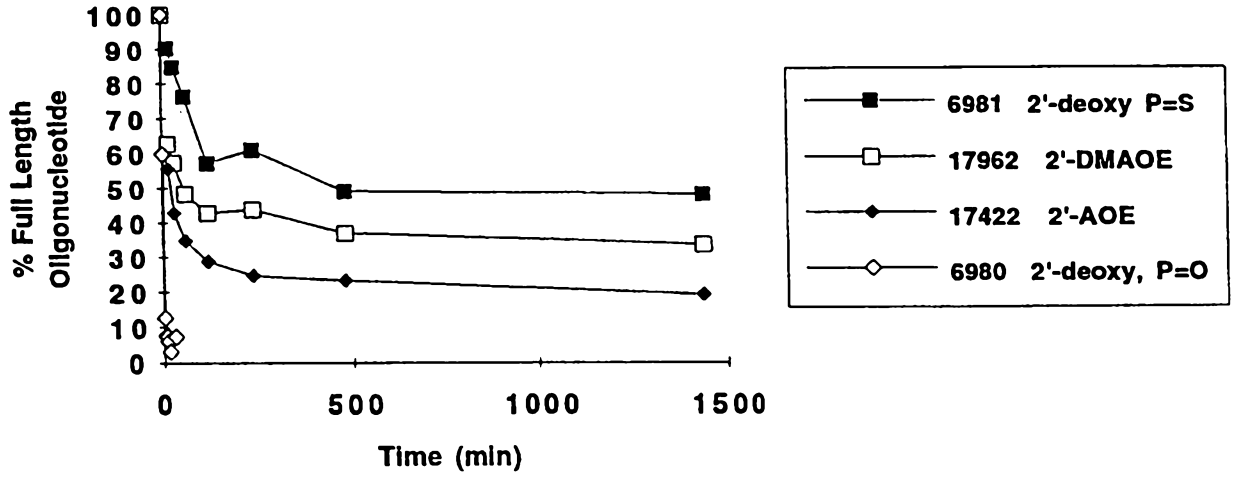
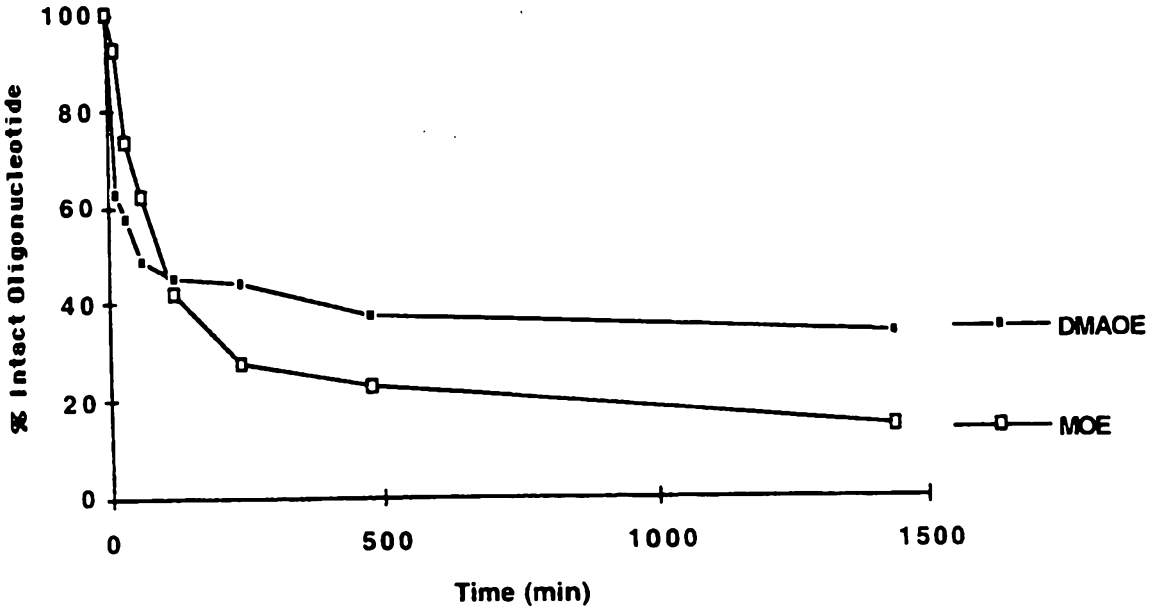

% Full Length Oligonucleotide
100
90
80
70
60
50
40
30
20
10
0
0
500
1000
1500
Time (min)
6981 2'-deoxy P=S
17962 2'-DMAOE
17422 2'-AOE
6980 2'-deoxy, P=O

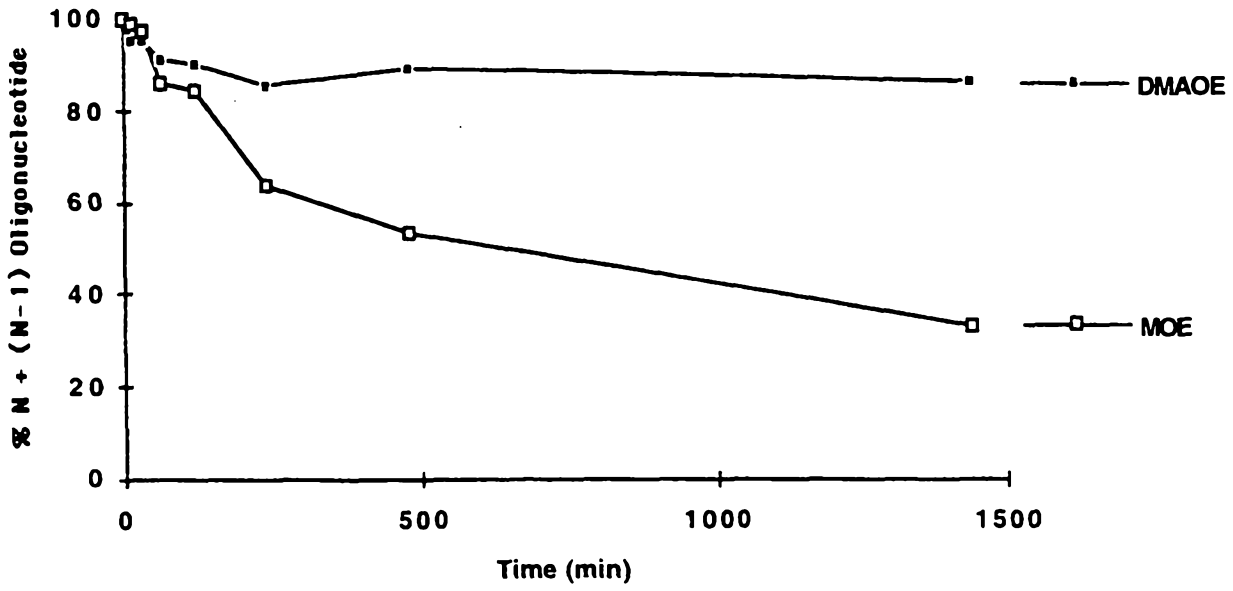
Figure 13



5' TTT TTT TTT TTT TTT* T*T*T* T 3'

Where T* is 2'-modified nucleotide (DMAOE or MOE)

Figure 14



5' TTT TTT TTT TTT TTT* T*T*T* T 3'

Where T* is 2'-modified nucleotide (DMAOE or MOE)

Figure 15

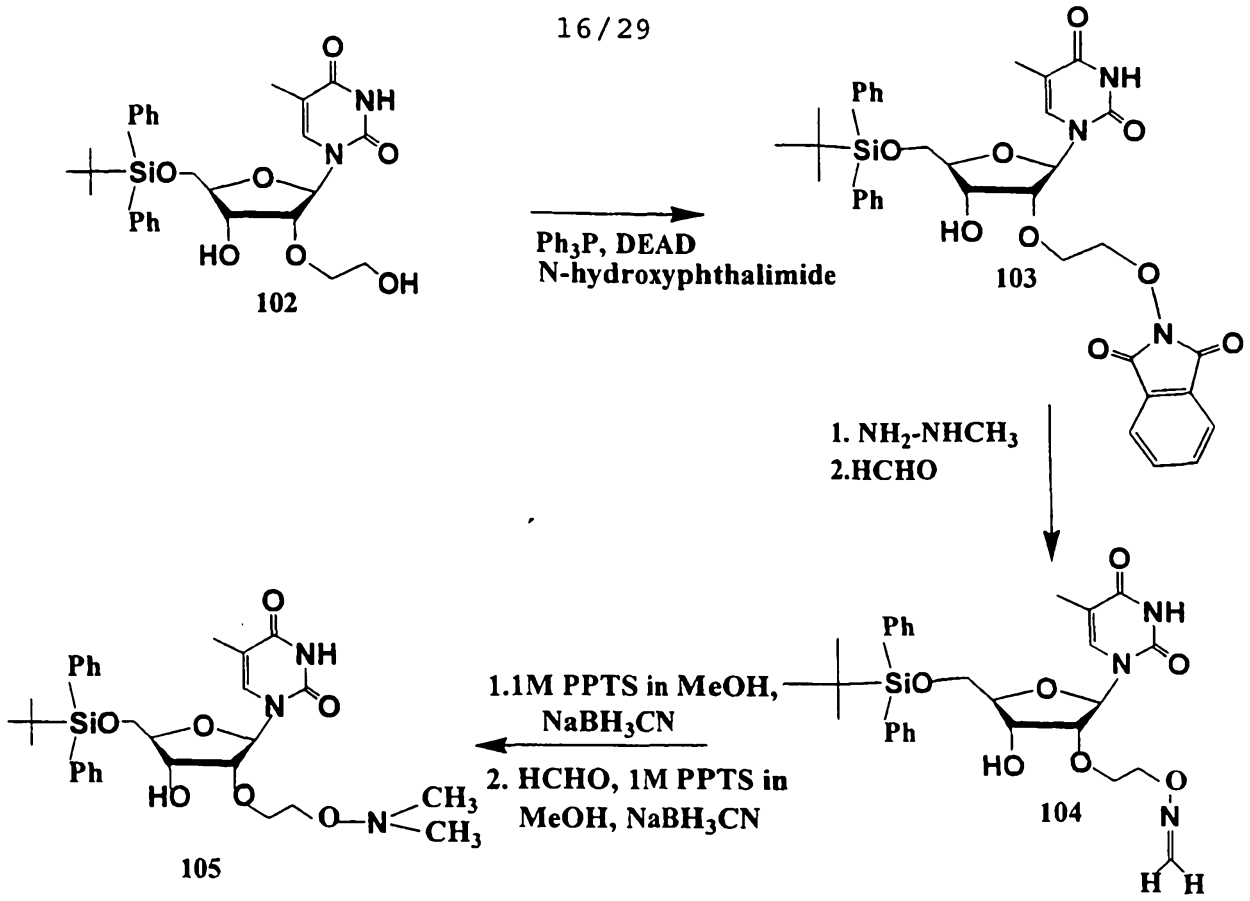

Ph
SiO
Ph
HO
O
OH
102
Ph3P, DEAD
N-hydroxyphthalimide
103
1. NH2-NHCH3
2.HCHO
104
1.1M PPTS in MeOH,
NaBH3CN
2. HCHO, 1M PPTS in
MeOH, NaBH3CN
105
CH3
CH3

Figure 16

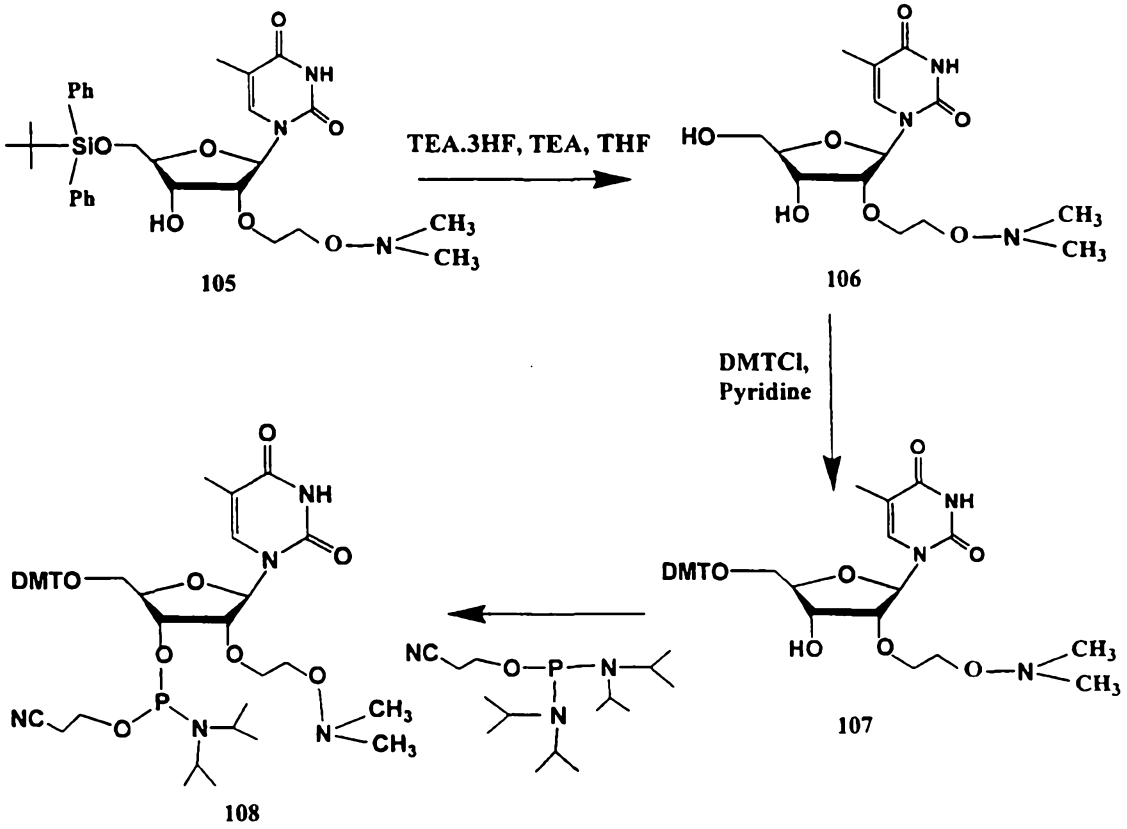

Ph
SiO
Ph
HO
TEA.3HF, TEA, THF
HO
HO
DMTCl,
Pyridine
DMTO
DMTO
NC
105
106
107
108

Figure 17

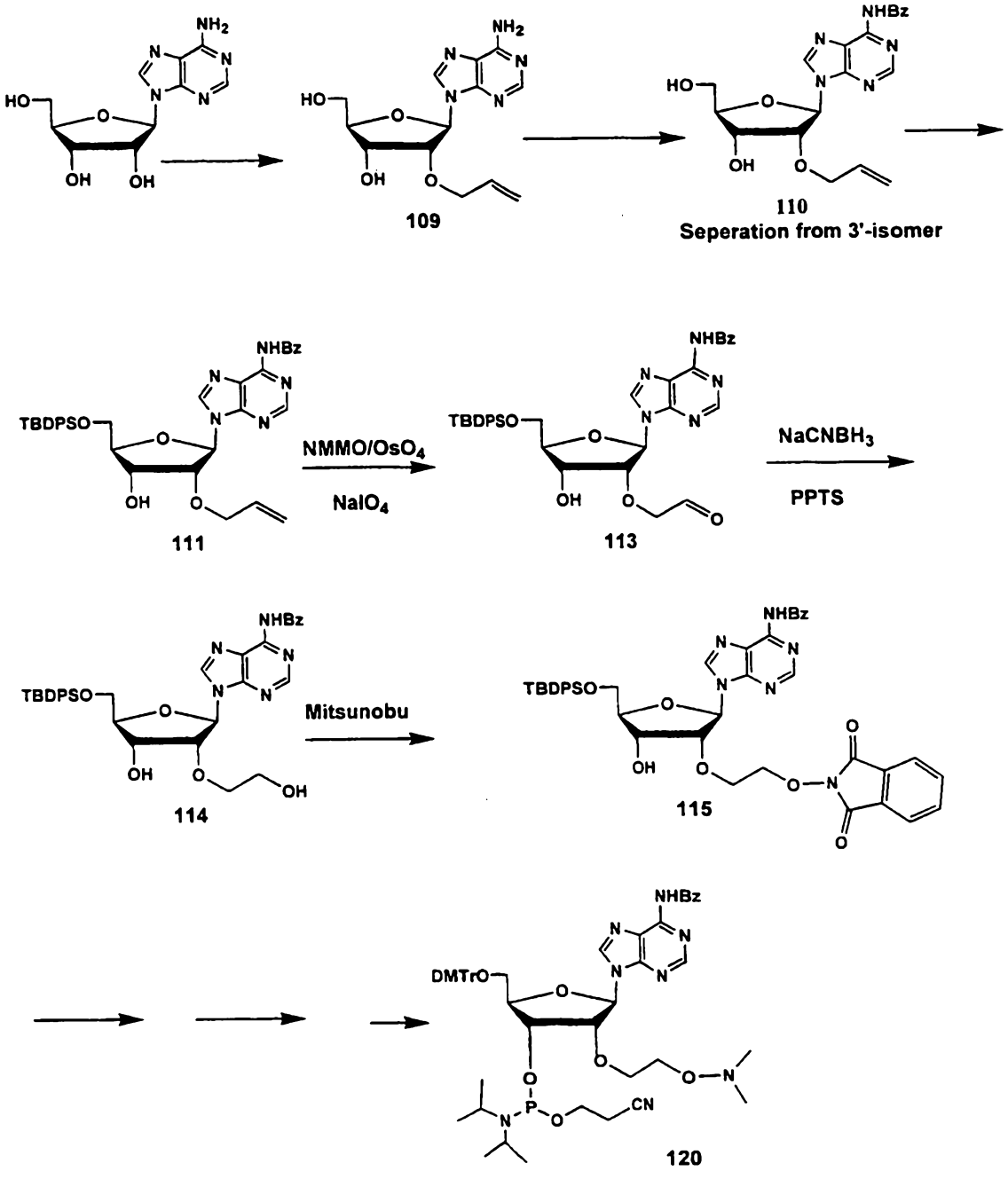

NH2
HO
OH
OH
109
110
Seperation from 3'-isomer
NHBz
TBDPSO
NMMO/OsO4
NaIO4
111
113
NaCNBH3
PPTS
Mitsunobu
114
115
DMTrO
CN
120

Figure 18

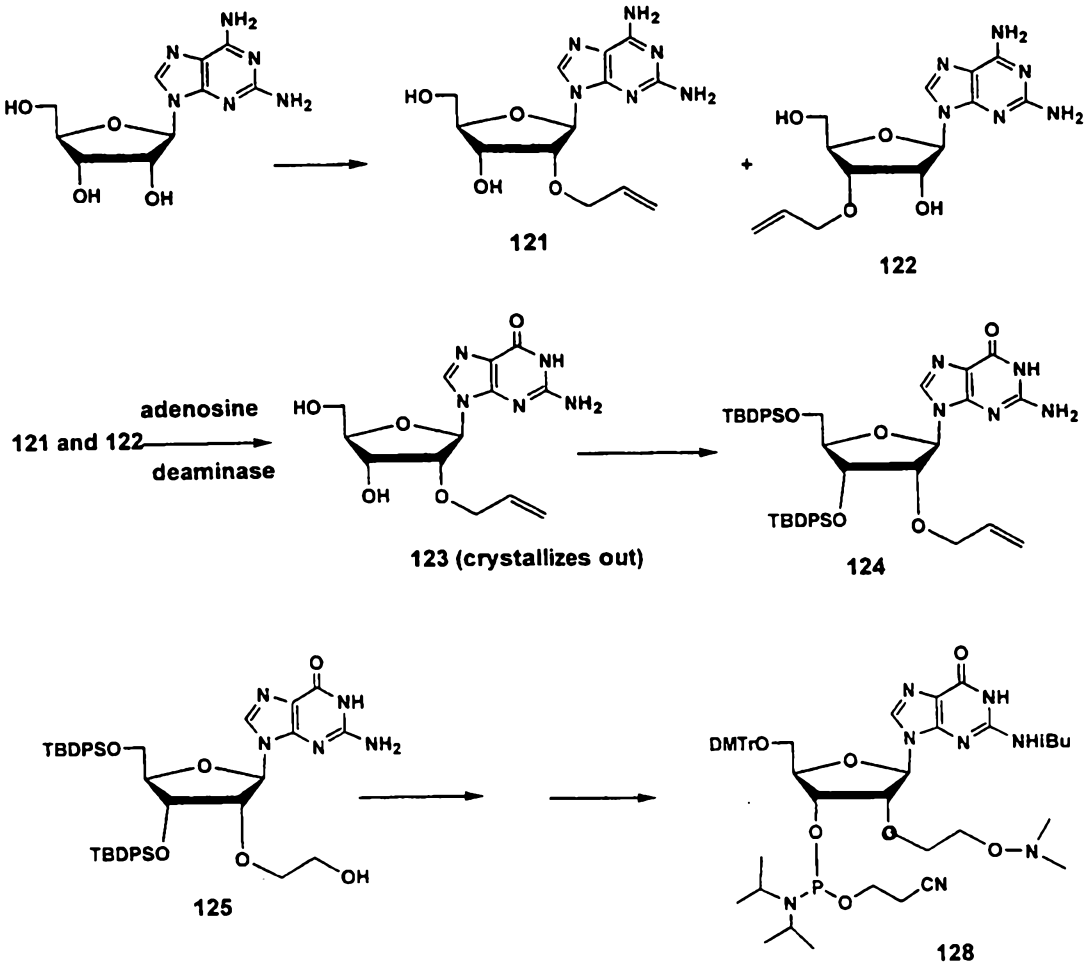

121
122
adenosine
121 and 122
deaminase
123 (crystallizes out)
124
125
128

Figure 19

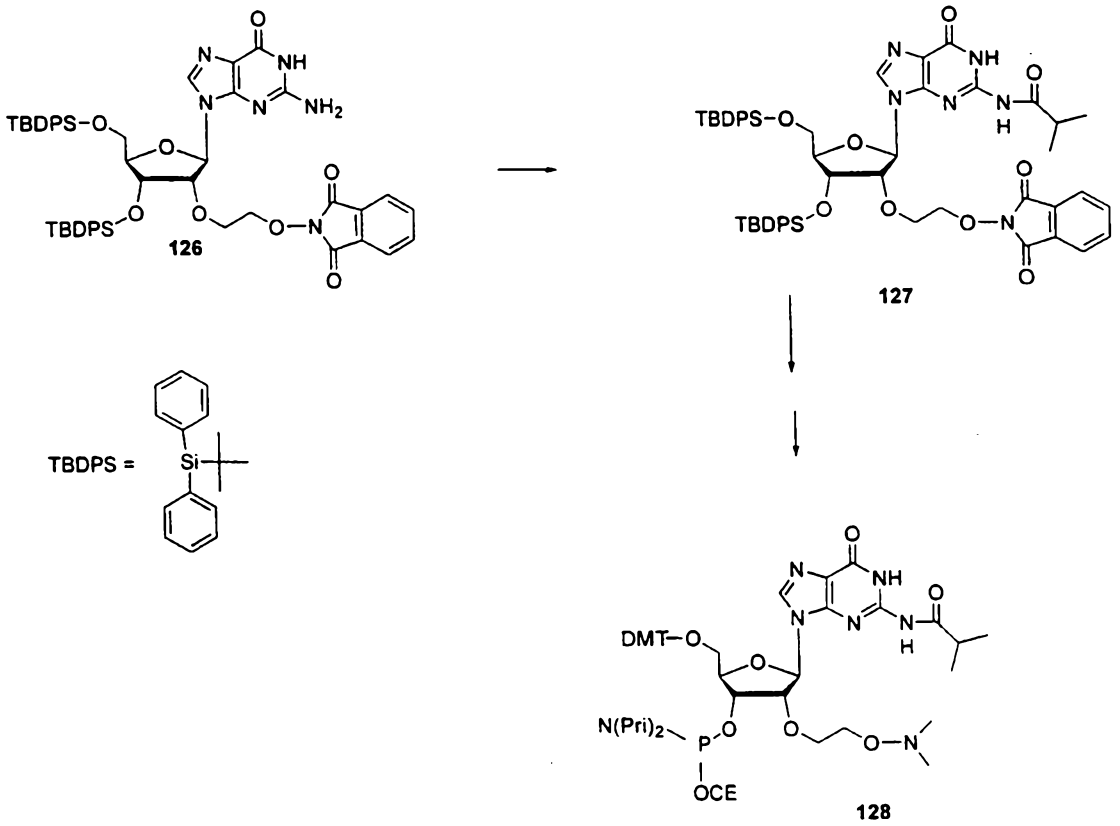


Figure 20

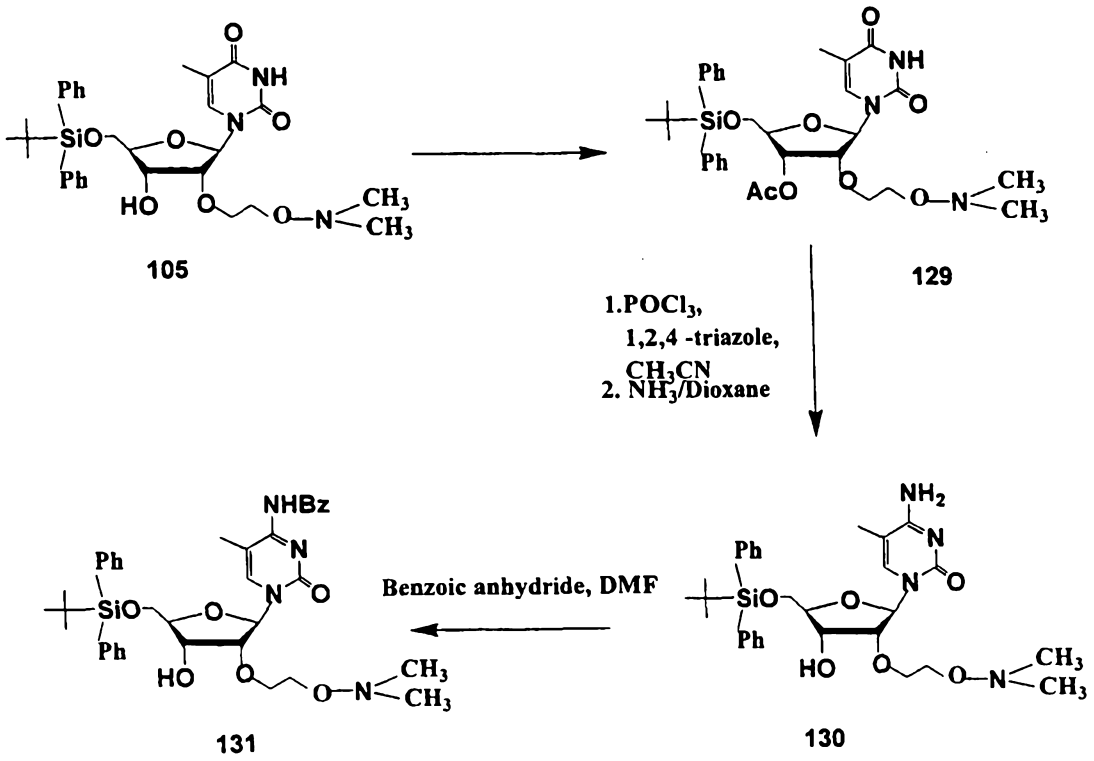

105
129
1.POCl3,
1,2,4 -triazole,
CH3CN
2. NH3/Dioxane
131
Benzoic anhydride, DMF
130

Figure 21

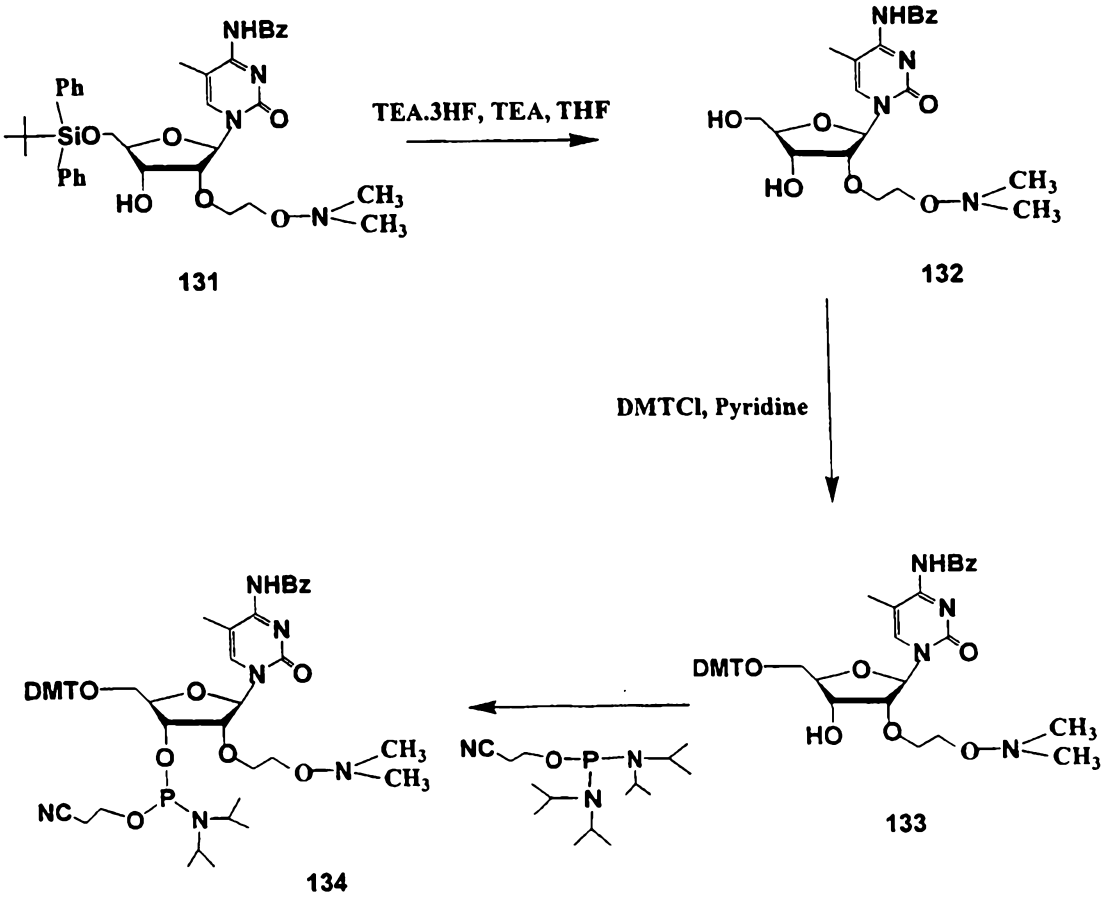

NHBz
Ph
SiO
Ph
HO
TEA.3HF, TEA, THF
131
132
DMTCl, Pyridine
DMTO
NC
133
134

Figure 22

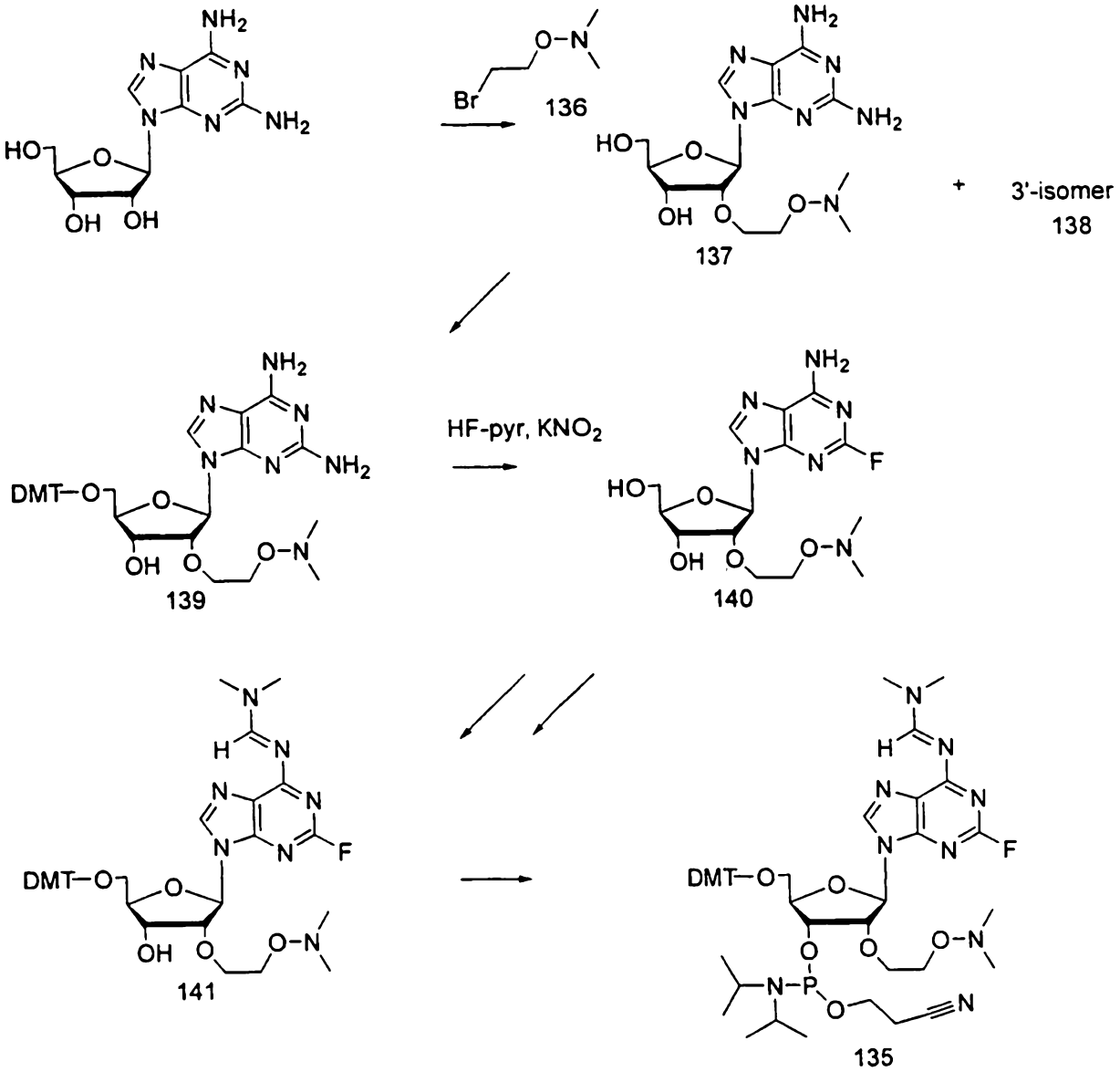

NH2
HO
OH OH
Br
136
137
+ 3'-isomer
138
HF-pyr, KNO2
DMT—O
139
140
141
135
F
N
O
H

Figure 23

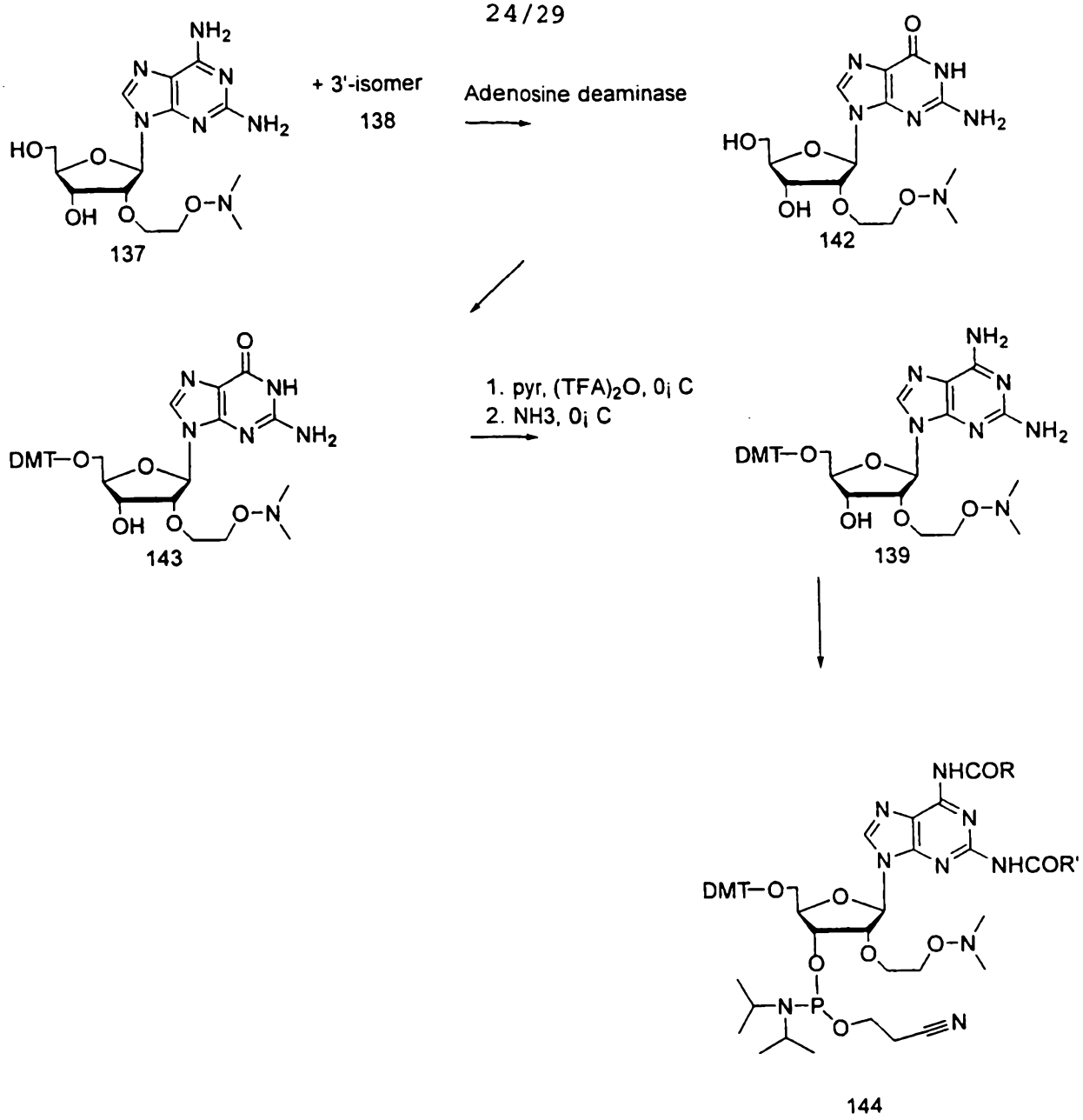

+ 3'-isomer
138
Adenosine deaminase
137
142
1. pyr, (TFA)2O, 0i C
2. NH3, 0i C
143
139
144

Figure 24

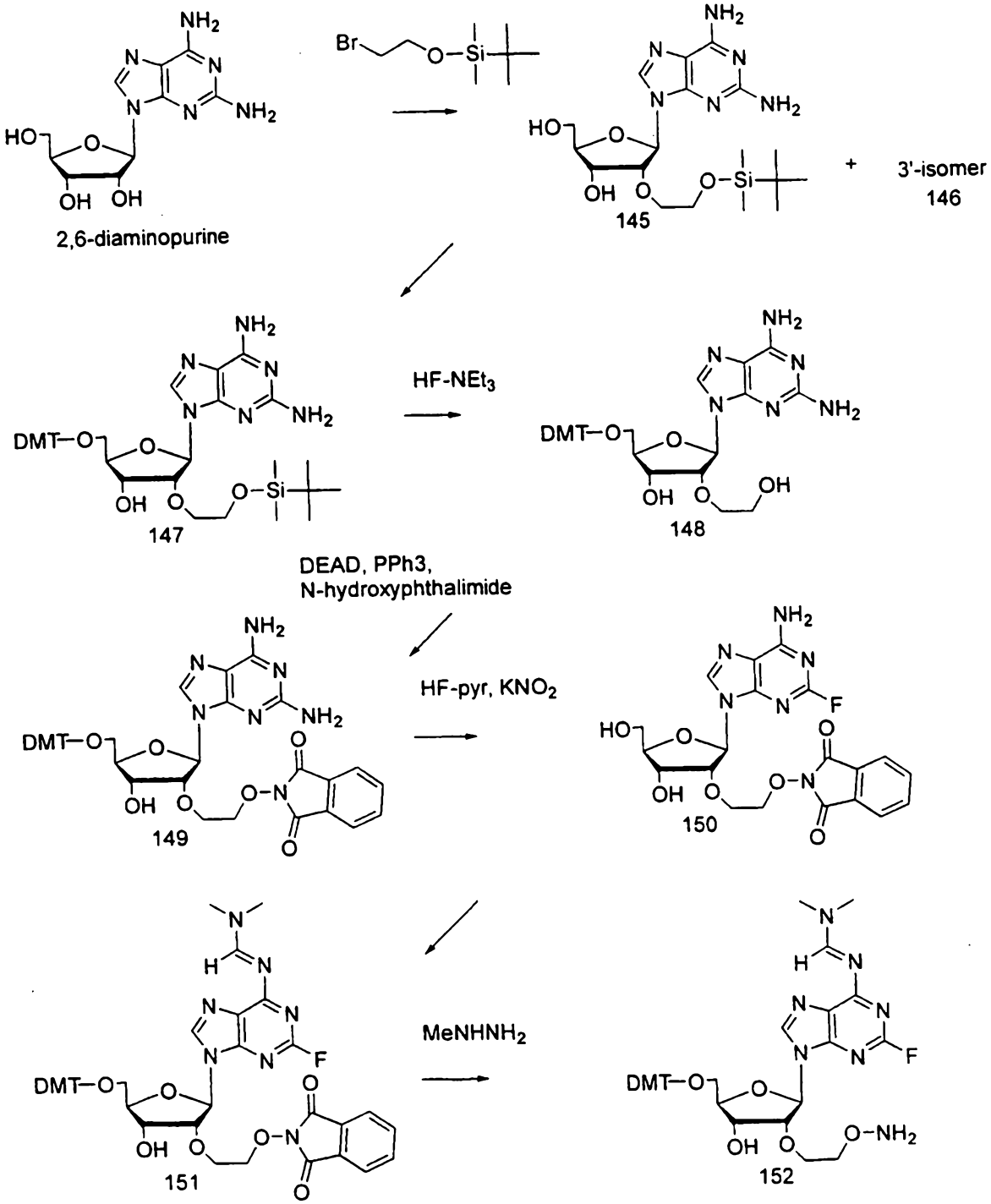

2,6-diaminopurine
145
+ 3'-isomer
146
147
HF-NEt3
148
DEAD, PPh3,
N-hydroxyphthalimide
149
HF-pyr, KNO2
150
151
MeNHNH2
152

Figure 25

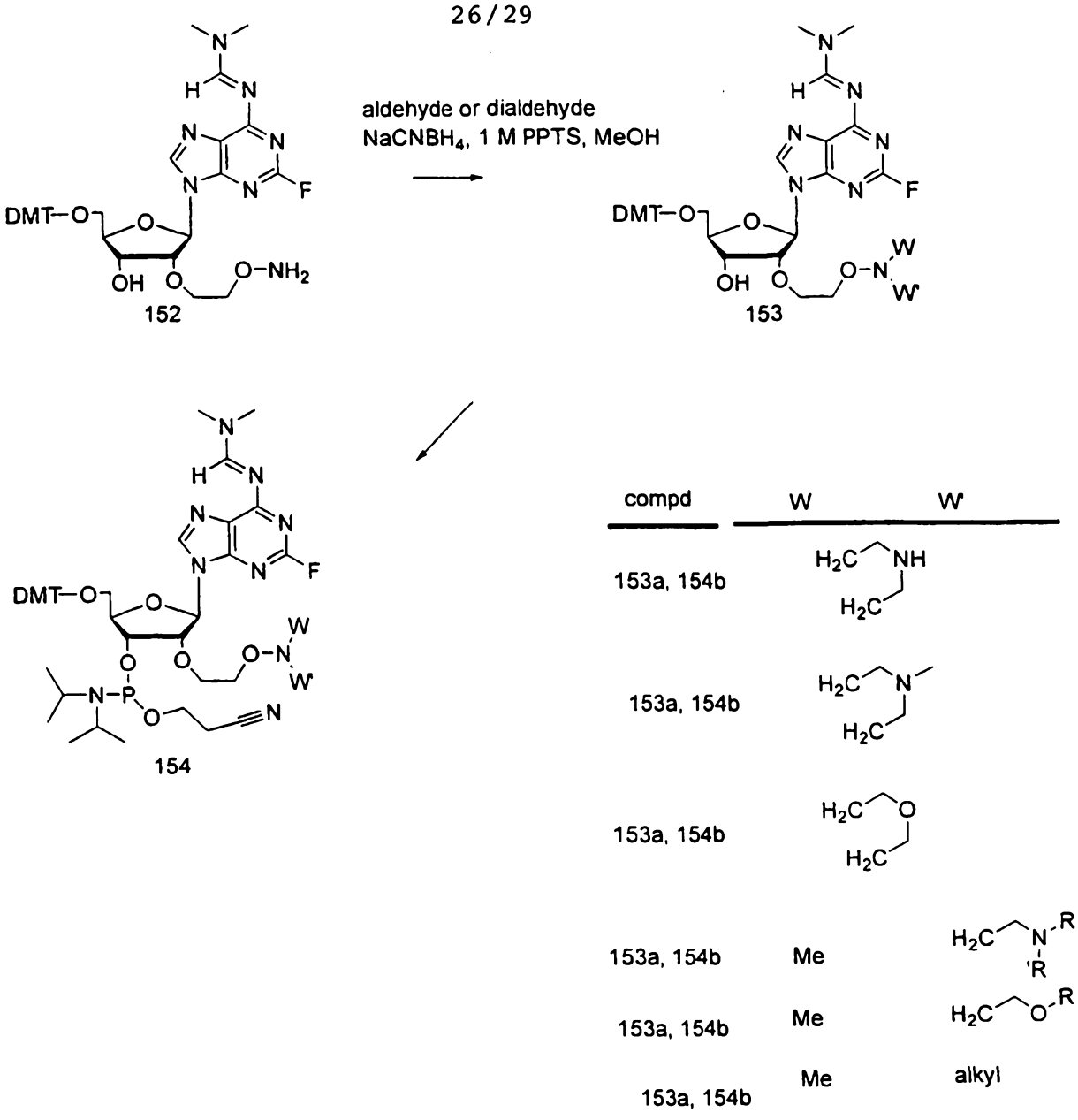

aldehyde or dialdehyde
NaCNBH4, 1 M PPTS, MeOH
DMT–O
OH
O–NH2
152
W
W'
153
154
compd
W
W'
153a, 154b
153a, 154b
153a, 154b
153a, 154b
Me
153a, 154b
Me
153a, 154b
Me
alkyl

Figure 26

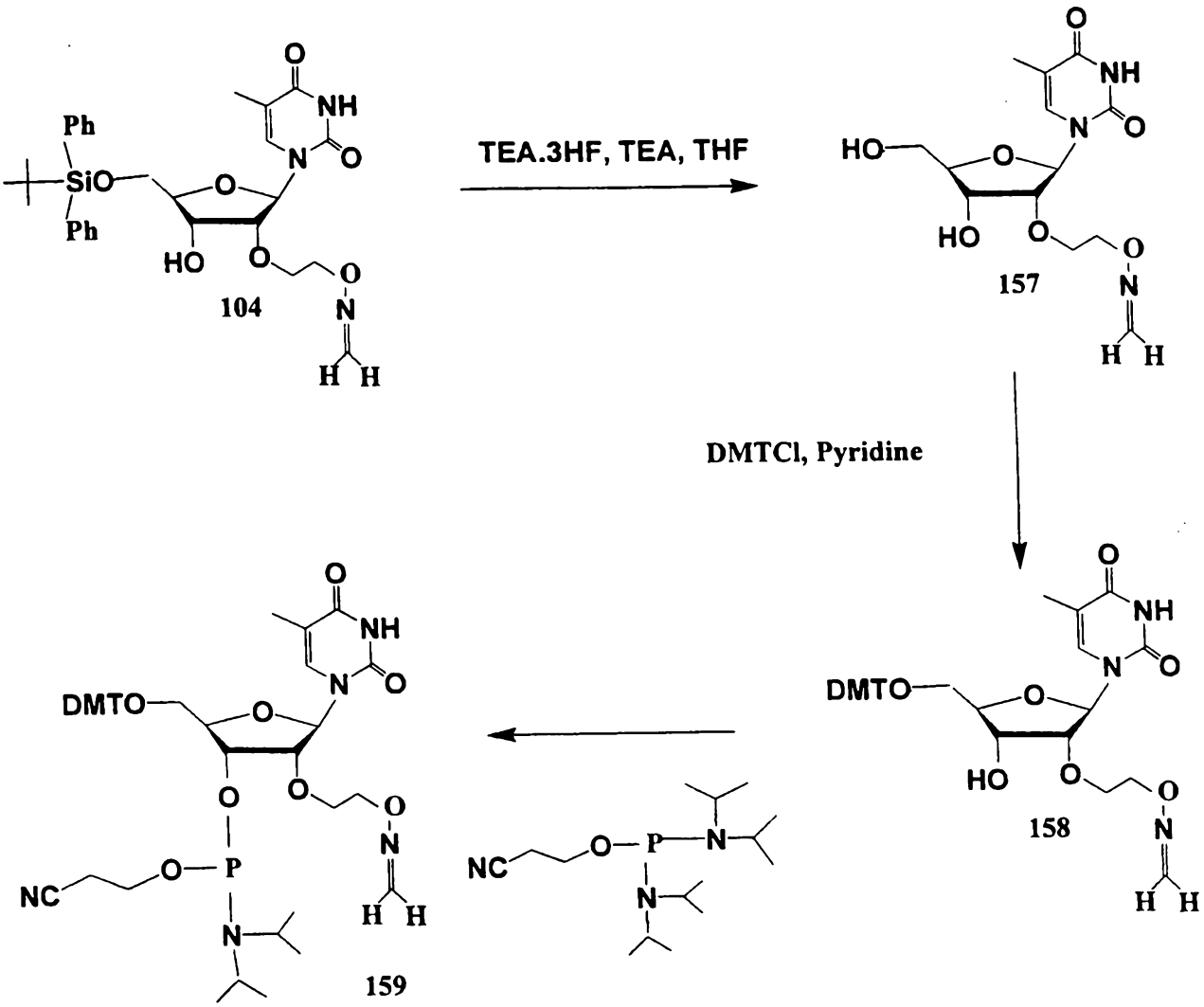

Ph
SiO
Ph
HO
104
TEA.3HF, TEA, THF
HO
157
DMTCl, Pyridine
DMTO
158
DMTO
NC
159

Figure 27

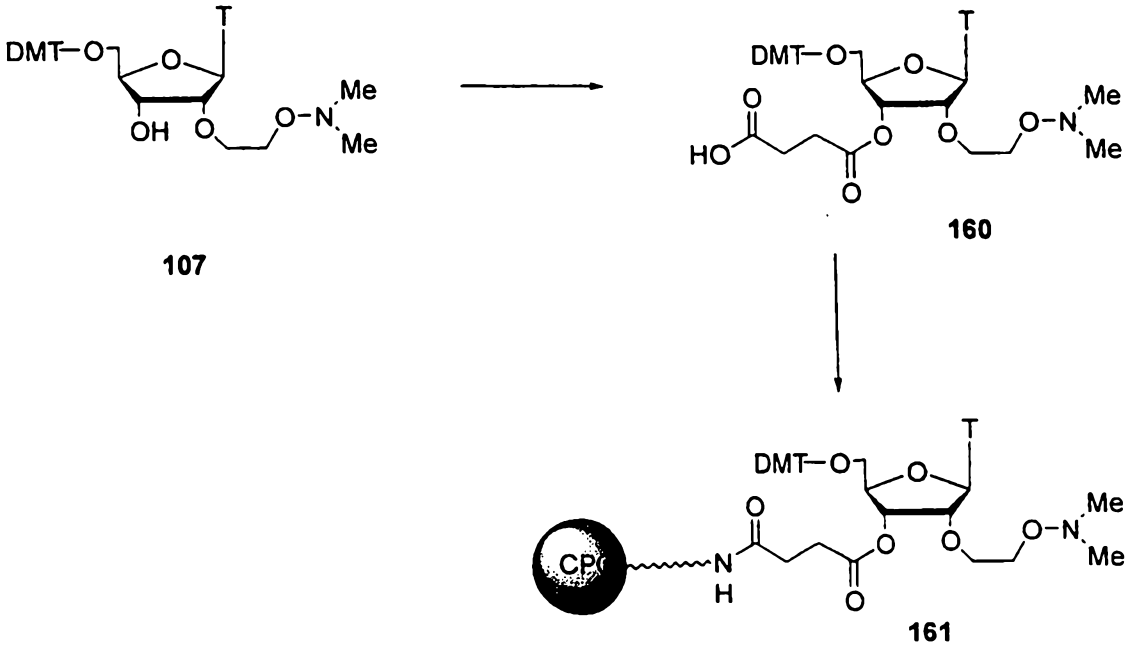
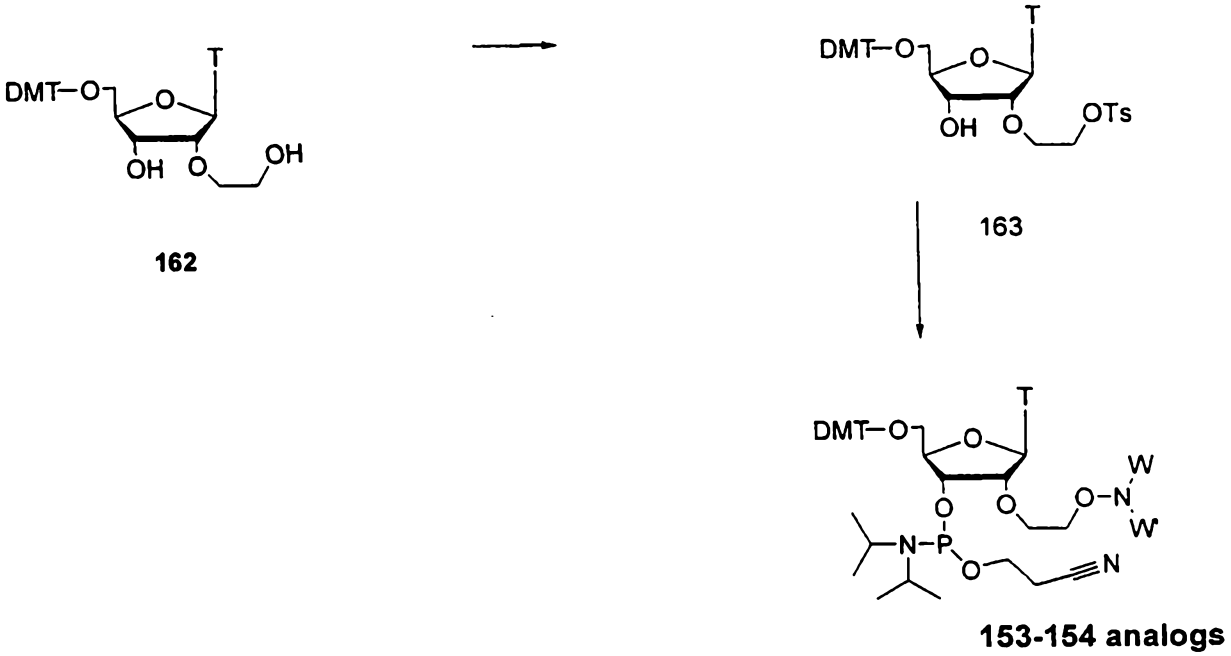
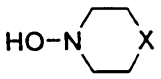


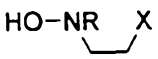
Figure 28

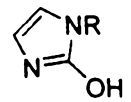


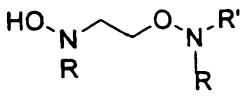
Amino-Oxy Precursursors:

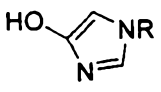


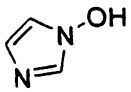
X = NH, O, S, N-alkyl











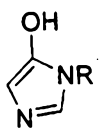


Figure 29