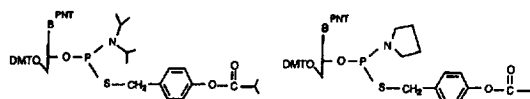
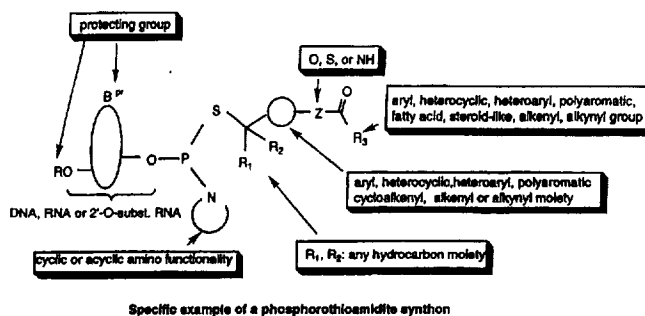




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07H 21/00, 19/10, 19/20	A1	(11) International Publication Number: WO 98/08858 (43) International Publication Date: 5 March 1998 (05.03.98)
(21) International Application Number: PCT/US97/14962 (22) International Filing Date: 26 August 1997 (26.08.97) (30) Priority Data: 60/024,537 26 August 1996 (26.08.96) US (71) Applicant: HYBRIDON, INC. [US/US]; 620 Memorial Drive, Cambridge, MA 02139 (US). (72) Inventor: IYER, Radahakrishnan, P.; 34 Shrewsbury Green Drive, Shrewsbury, MA 01545 (US). (74) Agents: KEOWN, Wayne, A. et al.; Hale and Dorr LLP, 60 State Street, Boston, MA 02109 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>

(54) Title: IMPROVED REAGENTS AND PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES CONTAINING PHOSPHORODITHIOATE INTERNUCLEOSIDE LINKAGES

**(57) Abstract**

The invention provides new methods for synthesizing oligonucleotides containing at least one, and preferably all phosphorodithioate internucleoside linkages with less than 5 %, and preferably less than 2 %, and preferably less than 1 % and most preferably undetectable amounts of phosphoromonothioate contamination. This level of purity in the synthesis of phosphorodithioates has previously been very difficult to achieve with all existing methods. The invention further provides phosphorothioamidite nucleoside synthons comprising a sulfur protecting group that is stable under normal oligonucleotide synthesis conditions.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

IMPROVED REAGENTS AND PROCESS FOR SYNTHESIS OF
OLIGONUCLEOTIDES CONTAINING PHOSPHORODITHIOATE
INTERNUCLEOSIDE LINKAGES

5

Field of the Invention

The invention relates to the chemical synthesis of oligonucleotides and to chemical entities useful in such synthesis.

10

Background of the Invention:

Oligonucleotides have become indispensable tools in modern molecular biology, being used in a wide variety of techniques, ranging from diagnostic probing methods to PCR to antisense inhibition of gene expression. This widespread use of oligonucleotides has led to an increasing demand for rapid, inexpensive and efficient methods for synthesizing oligonucleotides.

The synthesis of oligonucleotides for antisense and diagnostic applications can now be routinely accomplished. See *e.g.*, *Methods in Molecular Biology*, vol. 20: *Protocols for Oligonucleotides and Analogs*, pp. 165-189 (S. Agrawal, Ed., Humana Press, 1993); *Oligonucleotides and Analogues: A Practical Approach*, pp. 87-108 (F. Eckstein, Ed., 1991); and Uhlmann and Peyman, *supra*. Agrawal and Iyer, *Curr. Op. in Biotech*, vol. 6:12, 1995; and *Antisense Research and Applications* (Crooke and Lebleu, Eds., CRC Press, Boca Raton, 1993). Early synthetic approaches included phosphodiester and phosphotriester chemistries. Khorana et al., *J. Molec. Biol.*, vol. 72:209, 1972, discloses phosphodiester chemistry for oligonucleotide synthesis. Reese, *Tetrahedron Lett.*, vol. 34:3143-3179 (1978), discloses phosphotriester chemistry for synthesis of oligonucleotides and polynucleotides. These early approaches have largely given way to the more efficient phosphoramidite and H-phosphonate approaches to

synthesis. Beaucage and Caruthers, *Tetrahedron Lett*, vol. 22:1859-1862 (1981), discloses the use of deoxynucleoside phosphoramidites in polynucleotide synthesis. Agrawal and Zamecnik, US Patent No. 5,149,798 (1992), discloses optimized synthesis of oligonucleotides by the H-phosphonate approach.

5 Both of these modern approaches have been used to synthesize oligonucleotides having a variety of modified internucleotide linkages. Agrawal and Goodchild, *Tetrahedron Lett.*, vol. 28:3539-3542 (1987), teaches synthesis of oligonucleotide methylphosphonates using phosphoramidite chemistry. Connolly et al., *Biochemistry*, vol. 23:3443 (1984), discloses synthesis of oligonucleotide phosphorothioates using
10 phosphoramidite chemistry. Jager et al., *Biochemistry*, vol. 27:72 (1988), discloses synthesis of oligonucleotide phosphoramidates using phosphoramidite chemistry. Agrawal et al., *Proc. Ant. Acad. Sci. USA*, vol. 85:7079-7083 (1988), discloses synthesis of oligonucleotide phosphoramidates and phosphorothioates using H-phosphonate chemistry.

15 Synthesis of certain types of modified oligonucleotides remains problematic, however. For example, synthesis of oligonucleotides containing phosphorodithioate internucleoside linkages has been plagued by oxidation of the phosphorodithioate internucleoside linkage to a phosphorothioate internucleoside linkage. Using a β -cyanothyl group to protect the sulfur moiety from oxidizing during the
20 phosphorodithioate synthesis as been used in a previous approach with limited success and has resulted in high levels 8-9% of contaminating phosphoromonothioate incorporated into the phosphorodithioate product (Dahl et al, *Acta Chem. Scand.* 1989, 43, 896-901; Dahl et al, *Tetrahedron Lett.* 1990, 31, 3489-3492; Bjergarde et al, *Nucleic Acid Res.* 1991, 19, 5843-5850). Beaton et al, *Oligonucleotides and Analogues: A*
25 *practical approach*; Eckstein, Ed.; IRL Press 1991;pp 109-135, discloses an improved procedure for synthesizing phosphorodithioate oligonucleotides. However, even in this procedure, at least 2-4% of the phosphorodithioate internucleoside linkages were oxidized to phosphorothioates and moreover, the synthons containing the 2,4, dichlorobenzyl group used to block the sulfure are highly unstable to oxidation and does

not remain stable through the course of a single synthesis. Wiesler and Caruthers, *J. Org. Chem.*, 1996, 61, 4272-4281, disclose yet another improved procedure for phosphorodithioate synthesis, however this procedure is also plagued with 2-5% phosphoromonothioate contamination incorporated into the phosphorodithioate oligonucleotide product.

One attractive feature of the phosphorodithioate internucleoside linkage is that it is achiral. Thus, this internucleoside linkage can be used to make oligonucleotides which are stereochemically pure. In contrast, the phosphorothioate internucleoside linkage exists as R_p and S_p enantiomers. Thus, oligonucleotides containing phosphorothioate internucleoside linkages exist as racemic mixtures which contain 2^n stereoisomerically distinct species, wherein n represents the number of phosphorothioate internucleoside linkages present in the oligonucleotide. Accordingly, even low levels of oxidation of the phosphorodithioate internucleoside linkages to phosphorothioates can convert a stereochemically pure oligonucleotide preparation to a relatively complex racemic mixture.

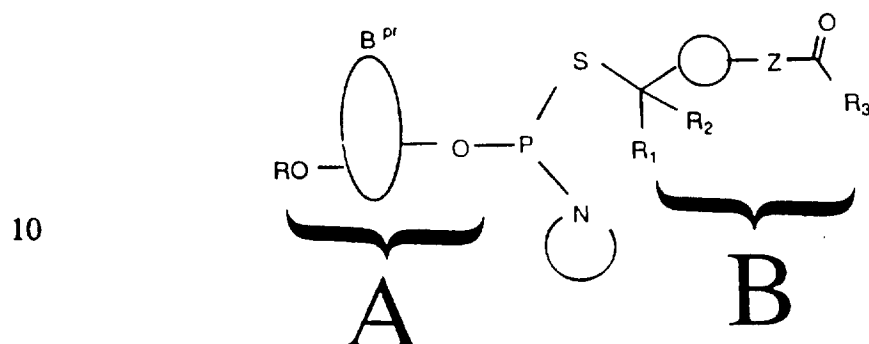
There is, therefore, a need for improved reagents and processes for phosphorodithioate oligonucleotide synthesis. Ideally, such reagents and processes should be suitable for use in existing oligonucleotide synthesis protocols. There is further a need for oligonucleotides having exclusively phosphorodithioate internucleoside linkages as well as for oligonucleotides which contain phosphorothioate linkages.

Summary of the Invention

The invention provides new methods for synthesizing oligonucleotides containing phosphorodithioate internucleoside linkages with less than 5%, and preferably less than 2% phosphoromonothioate contamination. This level of purity in the synthesis of phosphorodithioates has previously been very difficult to achieve with all existing methods. Thus, the invention also provides a population of phosphorodithioate-containing oligonucleotides with less than 5% and preferably less than 2% contaminating phosphoromonothioate incorporated therein. Additionally, the invention further provides

nucleoside synthons for phosphorodithioate synthesis having a sulfur protecting group that is stable under normal oligonucleotide synthesis conditions.

One aspect of the invention provides novel synthons for phosphorodithioate synthesis comprising a sulfur protecting group (shown as "B"), the monomer synthon
5 having the general structure I:



wherein \bigcirc is aryl, heterocyclic, heteroaryl, polyaromatic, cycloalkenyl, alkenyl,
15 alkynyl; R_3 is aryl, heterocyclic heteroaryl, polyaromatic, alkenyl and substituted
derivatives thereof, alkynyl or combinations of alkenyl moieties and alkynyl moieties; X
is S, NH or O; R_1 and R_2 may be the same or different hydrocarbon moiety; R is a 5'OH
blocking group (see e.g. Sonveaux in Methods in Molecular Biology Vol 26: Protocols
for Oligonucleotide Conjugates pp 28-36 (S Agrawal, Ed., Humana Press, 1994),
20 preferably dimethoxytrityl or trityl, B is a nucleoside base; P^r is a base protecting group,
such as any suitable base labile group as is known in the art for the protection of
exocyclic amino groups such as benzoyl groups or isobutyryl groups; A represents a
nucleoside moiety which is a deoxyribonucleoside, a ribonucleoside, a substituted
ribonucleoside wherein the 2' position of the ribose moiety is substituted with an O-lower
25 alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or
O-allyl group having 2-6 carbons atoms wherein such alkyl, aryl or allyl group may be
unsubstituted or may be substituted, e.g., with halo hydroxy, trifluoromethyl, cyano, nitro,
acyl, acyloxy, alkoxy carboxyl, carbalkoxyl or amino groups; and \bigcirc^N represents a

cyclic or acyclic amino functionality. The sulfur protecting group is represented as B. Such monomer synthons are suitable for use in the well known phosphoramidite synthetic chemistries.

The configuration of structure 1 and the preferred embodiments thereof are particularly advantageous for effectively preventing oxidation of the sulfur during oligonucleotide phosphorodithioate synthesis, thereby preventing undesirable phosphoromonothioate contamination of the synthesis product. Thus, in another aspect, the invention provides a method for synthesizing oligonucleotides comprising phosphorodithioate internucleotide linkages at a higher purity level than has previously been achieved. This new method comprises sequentially coupling nucleoside monomer synthons of the invention having the sulfur protecting groups in accordance with the invention to produce a sulfur protected oligonucleotide, followed by a sulfurization step with any suitable sulfurizing reagent as is well known in the synthesis of oligonucleotides, and after the desired nucleotide length is reached, deprotection of the base protecting group and optionally, at the same time, deprotection of the sulfur protecting group with a chemoselective removing reagent. The method according to the invention preferably utilizes the well known phosphoramidite chemistries. In another embodiment of the invention the same protected phosphorodithioate moiety may be generated using H-phosphonate chemistry, and subsequently deprotected to the desired phosphorodithioate free of contaminating phosphoromonothioate.

The use of these novel reagents and methods of the invention provides many advantages. For example, phosphorodithioate containing oligonucleotides are synthesized with almost no phosphoromonothioate (chirally mixed) contamination resulting in the synthesis of oligonucleotides comprising internucleotide linkages which are achiral. Such achiral phosphorus internucleotide linkages have been found to be very advantageous when incorporated into antisense oligonucleotides for therapeutic use as they are highly stable toward both chemical and enzymatic hydrolysis. Moreover, studies have indicated that phosphorodithioate oligonucleotides have biophysical and biological properties which make them ideally suited for antisense applications, including but not

limited to the activation of RNase H which is believed to be essential for antisense activity *in vivo*.

In addition, the reagents and methods of the invention may be used to produce a population of oligonucleotides comprising mixed chemical backbones such as a combination of one or more linkages of phosphorodithioate, methylphosphonate phosphorothioate or phosphodiester wherein the oligonucleotide population comprises less than 2% contaminating phosphoromonothioate incorporated therein.

Oligonucleotides comprising such mixed backbones may be synthesized in order to maintain the overall advantages provided by the achiral nature of the phosphorodithioate linkage coupled with a stereocontrolled synthesis of the P-chiral phosphorothioate or P-chiral methylphosphonate linkages which make up the mixed backbone of the desired oligonucleotide.

Brief Description of the Drawing:

Fig. 1 is a schematic of the general structure of the phosphorothioamidite synthon of the invention with the sulfur protecting group as well as examples of the structures of specific phosphorothioamidite synthons in accordance with the invention.

Fig. 2 shows the scheme for the synthesis of a phosphorothioamidite synthon of the invention as described in Example 1.

Fig. 3 shows the scheme for the synthesis of a thiaphospholidine synthon of the invention as described in Example 2.

Fig. 4 shows the synthesis of dinucleoside phosphorodithioates using a phosphorothioamidite synthon of the invention as is described in Example 3.

Detailed Description of the Invention

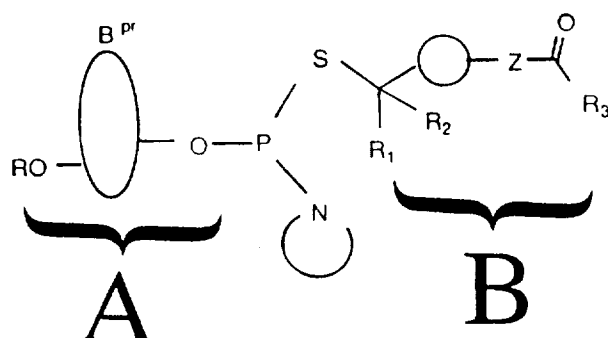
The invention relates to the chemical synthesis of oligonucleotides containing phosphorodithioates and to chemical entities useful in such synthesis and the phosphorodithioate-containing oligonucleotide product of such synthesis. The patents

and publications identified in this specification are within the knowledge of those skilled in this field and are hereby incorporated by reference in their entirety.

The invention provides new methods for synthesizing oligonucleotides containing at least one, and preferably all phosphorodithioate internucleoside linkages with less than 5%, and preferably less than 2%, and preferably less than 1% and most preferably undetectable amounts of phosphoromonothioate contamination. This level of purity in the synthesis of phosphorodithioates has previously been very difficult to achieve with all existing methods. The invention further provides phosphorothioamidite nucleoside synthons comprising a sulfur protecting group that is stable under normal oligonucleotide synthesis conditions.

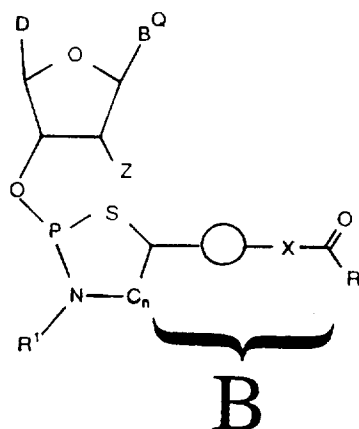
For the purposes of the invention, the term "oligonucleotide" includes polymers of two or more deoxyribonucleotide, ribonucleotide or 2'-O-substituted ribonucleotide monomers, or any combination thereof. In certain embodiment oligonucleotides will preferably have from about 5-50 nucleotides preferably about 10-45 nucleotides, preferably about 12-40 nucleotides, preferably about 15-35 nucleotides. In embodiments of oligonucleotides according to the invention that have fewer than all phosphorodithioate linkages, the other internucleoside linkages may be of any of the known internucleoside linkages, and are preferably also achiral linkages or chirally pure linkages. In certain preferred embodiments these internucleoside linkages may be P-chiral phosphorothioate, phosphodiester, or P-chiral methylphosphonate linkages. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents including without limitation lipophilic groups, intercalating agents, diamines and adamantane. For purposes of the invention, the term 2'-O-substituted means substitution of the 2' position of the pentose moiety with an -O- lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an O-aryl or allyl group having 2-6 carbon atoms wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano nitro, acyl, acyloxy, alkoxy carboxyl, cabalkoxyl or amino groups.

The phosphorothioamidite synthons in accordance with the invention have the following general structure I:



wherein \bigcirc is aryl, heterocyclic, heteroaryl, polyaromatic, cycloalkenyl, alkenyl, alkynyl; R_3 is aryl, heterocyclic heteroaryl, polyaromatic, alkenyl and substituted derivatives thereof, alkynyl or combinations of alkenyl moieties and alkynyl moieties; X is S, NH or O; R_1 and R_2 may be the same or different hydrocarbon moiety; R is a 5'OH blocking group (see e.g. Sonveaux in Methods in Molecular Biology Vol 26: Protocols for Oligonucleotide Conjugates pp 28-36 (S Agrawal, Ed., Humana Press, 1994), preferably dimethoxytrityl or trityl, B is a nucleoside base; Pr is a base protecting group, such as any suitable base labile group as is known in the art for the protection of exocyclic amino groups such as benzoyl groups or isobutyryl groups; A represents a nucleoside moiety which is a deoxyribonucleoside, a ribonucleoside, a substituted ribonucleoside wherein the 2' position of the ribose moiety is substituted with an O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or O-allyl group having 2-6 carbons atoms wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, e.g., with halo hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy carboxyl, carbalkoxyl or amino groups; and \bigcirc^N represents a cyclic or acyclic amino functionality. The sulfur protecting group is shown in brackets as B. Such monomer synthons are suitable for use in the well known phosphoramidite synthetic chemistries.

In one preferred embodiment of the invention, the phosphorothioamidite synthons in accordance with the invention have the following general structure II:



wherein \bigcirc is aryl, heterocyclic, heteroaryl, polyaromatic, cycloalkenyl, alkenyl, alkynyl; R is aryl, heterocyclic heteroaryl, polyaromatic, alkenyl and substituted derivatives thereof, alkynyl or combinations of alkenyl moieties and alkynyl moieties; X is S, NH or O; n=1 or 0; R' is alkyl (1-15 carbons straight or branched), an aromatic or heterocyclic 5 or 6 membered ring comprising 1-5 heteroatoms, aralkyl or heteroaryl; B is a nucleoside base, D is a 5'OH blocking group (see *e.g.* Sonveaux in *Methods in Molecular Biology* vol 26: *Protocols for Oligonucleotide Conjugates* pp. 28-36 (S Agrawal, Ed., Humana Press, 1994), preferably dimethoxytrityl or trityl, Q is a base protecting group, such as any suitable base labile group as is known in the art for the protection of exocyclic amino groups such as benzoyl groups or isobutyryl groups; Z is H when the nucleoside is a deoxyribonucleoside is desired, Z is OH for a ribonucleoside, or Z is an O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or O-allyl group having 2-6 carbons atoms wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, *e.g.*, with halo hydroxy, trifluoromethyl, cyano, nitro, acyl acyloxy, alkoxy carboxyl, carbalkoxyl or amino groups (for a 2'-substituted ribonucleotide). The sulfur protecting group is indicated by B.

In a preferred embodiment of structure I or II, \bigcirc is a phenyl group, a cis or trans ethenyl, or thiozole and R is isopropyl phenyl, cis or trans ethenyl, thiozole, pyrrole or

furan. Specific, nonlimiting examples of suitable synthons of the invention are described in the examples and in the Figs.

In another preferred embodiment of structure I or II, Q is N-pent-4-enoyl ($\text{CH}_2=\text{CH}(\text{CH}_2)_2\text{CO}$) (Iyer et al., *J.Org. Chem*, vol 60:8123-33 (1995)). This base protecting group referred to herein as PNT, is particularly preferred (see Fig. 2 for structure). This group may also be used to protect the 2' hydroxy moiety of the ribose group as well as the 2'-O-substituted moiety of the ribose unit of the nucleotide during synthesis of phosphorodithioate RNA or 2'-O-substituted RNA. A chemoselective removing agent may optionally be chosen such that it can simultaneously remove the base and RNA protecting group and the sulfur protecting group of the protected dithioate intermediate to yield the phosphorodithioate during synthesis. In another embodiment, the N-pent-4-enoyl or other base protecting group, may preferably be removed without removing the sulfur protecting group using a chemoselective removing agent such as Br_2 , Cl_2 and I_2 in pyridine/MeOH.

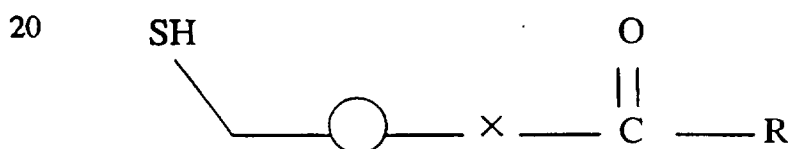
Schemes for the synthesis of such monomers according to the invention are described in the Examples and shown in Figs 1 and 2.

The sulfur-protected synthons of the invention can be synthetically prepared with less than 5%, and preferably less than 2% contamination from the desulfurized (oxidized) phosphoramidite without subjecting the synthesis product to further purification procedures. The synthon also remains highly stable to oxidation post-synthesis. Therefore, in yet another aspect, the invention provides a population of phosphorothioamidite synthons of structure I comprising less than 2%, and preferably comprising less than 1% and most preferably comprising undetected levels of desulfurized phosphoramidite contamination.

In another aspect of the invention, the invention provides a simple, highly efficient process for synthesizing an oligonucleotide comprising from one to about all phosphorodithioate linkages using well known synthesis procedures and the novel chemical entities of the invention. In one embodiment, this process comprises condensing in the presence of 1H-tetrazole an phosphorothioamidite nucleoside

comprising the sulfur protecting group of the invention, with another nucleoside, wherein at least one of the nucleosides has a nucleoside base-protective group, to produce adjacent nucleosides coupled by a phosphite linkage which is then sulfurized (*e.g.* elemental sulfur in pyridine/CS₂ or Beaucage reagent) to the protected phosphorodithioate, and eventually
 5 chemoselectively removing the sulfur protecting group preferably using a solution of K₂CO₃/MeOH.

Another embodiment comprises condensing in the presence of a suitable activator, such as pivaloyl chloride, a nucleoside H-thio-phosphonate with another nucleoside wherein at least one of the nucleosides has a base protecting group in accordance with an
 10 embodiment of the invention, to produce adjacent nucleosides coupled by a thio-H-phosphonate linkage, oxidizing the linkage in the presence of carbon tetrachloride with a protected thiol moiety in accordance with the invention (*e.g.* acyloxy-aryl mercaptan or heteroaryl mercaptan moiety as described above) to yield the protected dithioate, and removing, for example, acyloxy-aryl or heteroaryl sulfur protecting group of the invention
 15 as described above. Therefore another aspect of the invention is a method for synthesizing a phosphorodithioate oligonucleotide comprising the steps of: providing a thio-H-phosphonate nucleoside synthon; coupling in the presence of carbon tetrachloride, said synthon with a protected thiol moiety of the structure:



wherein \bigcirc is aryl, heterocyclic, heteroaryl, polyaromatic, cycloalkenyl, alkenyl,
 25 alkynyl; R is aryl, heterocyclic heteroaryl, polyaromatic, alkenyl and substituted derivatives thereof, alkynyl or combinations of alkenyl moieties and alkynyl moieties; X is S, NH or O, to yield a sulphur protected thio-H-phosphonate intermediate and coupling said intermediate with a second thio-H-phosphonate synthon.

Synthesis may be carried out in solution phase or on a solid support as is known in the art. For solid support synthesis, after conventional detritylation of a nucleoside linked to controlled pore glass (CPG) or any other suitable support such as polystyrene, a monomer synthon of the invention is coupled to the support linked monomer via conventional tetrazole activation for example. The resulting dinucleotide is oxidized with sulfur to the desired phosphorodithioate linkage. Unreacted CPG-bound nucleoside is then capped with acetic anhydride, for example, and the cycle is continued until the desired oligonucleotide length is achieved. simultaneously. The sulfur protecting group of the invention and the base protecting groups are removed as described above and optionally the oligonucleotide can simultaneously be cleaved from the solid support.

Each of the above methods for the synthesis of phosphorodithioate oligonucleotides yields product with less than 5% and preferably less than 2%, preferably less than 1% and most preferably undetectable levels of contaminating phosphoromonothioate-containing product prior to any further post-synthesis procedures. Therefore, in yet another aspect, the invention provides a population of phosphorodithioate-containing oligonucleotides comprising less than 5% and preferably less than 2%, and preferably comprising less than 1% and most preferably comprising undetected levels of contaminating phosphoromonothioate incorporated into the phosphorodithioate products.

In another preferred embodiment, oligonucleotides of mixed backbones may be prepared in accordance with the methods of the invention. Such oligonucleotides may comprise one or more phosphorodithioate linkages in combination with one or more phosphorothioate linkages, phosphodiester linkages or methylphosphonate linkages. If the phosphorothioate or methylphosphonate linkages are prepared in accordance with a stereocontrolled synthesis as is known in the art (see e.g. Iyer et al., *Tetrahedron Asymmetry*, 6: 1051-1054 (1995); and Stec et al., *Nucleic Acids Res*, vol. 19:5883-5888 (1991)), an oligonucleotide comprising both achiral linkages and chirally pure linkages is expected to have the same advantages as an oligonucleotide comprising all achiral linkages. For example, as an antisense therapeutic, an oligonucleotide comprising both

chirally pure methylphosphonate linkages and achiral phosphodithioate regions would maintain the consistent biochemical, biophysical and biological properties of a chirally pure methyl phosphonate but in addition with still activate Rnase H via the phosphorodithioate linkages and maintain the beneficial stability toward both chemical and enzymatic hydrolysis which renders the achiral phosphorodithioate linkage so desirable.

Mixed backbone oligonucleotides according to the invention, comprise a population of oligonucleotides having at least one and preferably a region of phosphorodithioate nucleotide linkages wherein less than 5% and preferably less than 2%, preferably less than 1% and most preferably undetectable amounts of contaminating phosphoromonothioate is incorporated into the oligonucleotide population. In some preferred embodiments of mixed backbone oligonucleotides according to the invention, several adjacent nucleosides comprising a first region of the oligonucleotide are connected by at phosphorodithioate linkages and several other adjacent nucleosides comprising a second region are connected by a different type of oligonucleotide linkage such as another achiral linkage or a chirally pure linkage such as a P-chiral phosphorothioate or P-chiral methylphosphonate. These preferred oligonucleotides are referred to herein as "chimeric oligonucleotides" or "chimeras". In certain particularly preferred chimeric oligonucleotides according to the invention, the oligonucleotides comprises a phosphorodithioate region, and a P-chiral methylphosphonate region. In this context, a "phosphorodithioate region" is a region within an oligonucleotide of from about 2 to about 15 contiguous nucleosides linked to each other through phosphorodithioate linkages. A "P-chiral methylphosphonate region" is a region within an oligonucleotide of from about 4 to about 20 contiguous nucleosides linked to each other through P-chiral methylphosphonate linkages. A phosphodiester region is a region within an oligonucleotide comprising from about 2 to about 15 contiguous nucleosides linked to each other through phosphodiester linkages. In a preferred chimeric oligonucleotide of the invention, the oligonucleotide comprises a phosphorodithioate region flanked on either side by a methyl phosphonate region, or alternatively, a methyl

phosphonate region flanked on either side by a phosphorodithioate region. In another preferred embodiment, a phosphodiester region is flanked on each side by a phosphorodithioate region or alternatively, a phosphorodithioate region is flanked on each side by a phosphodiester region.

5 Oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be labeled with a reporter group and used as probes in conventional nucleic acid hybridization assays. They can also be used as antisense “probes” of a specific gene in an experimental cell culture or animal system and to evaluate the effect of blocking such specific gene expression. This can be accomplished
10 by administering to a cell or an animal an oligonucleotide according to the invention that has a nucleotide sequence that is complementary to a specific gene that is expressed in the cell or animal to inhibit the expression of the specific gene, and observing the effect of inhibiting the expression of the specific gene. In this manner, oligonucleotides in accordance with the invention are preferable to traditional “gene knockout” approaches
15 because they are easier to use and can be used to block gene specific gene expression at selected stages of development for differentiation.

 Additionally, oligonucleotides according to the invention are useful in an antisense therapeutic approach. Therapeutic use of oligonucleotides according to the invention is for treating a disease caused by aberrant gene expression. This is
20 accomplished by administering to an individual having the disease a therapeutically effective amount of an oligonucleotide of the invention, wherein, the oligonucleotide is complementary to a gene that is aberrantly expressed, wherein such aberrant expression causes the disease. Preferably, such administration should be parenteral, oral, sublingual, transdermal, topical, intranasal or intrarectal. It may be desirable to administer
25 simultaneously or sequentially effective amount of one or more oligonucleotides of the invention as a single treatment episode.

 As antisense therapeutics, the achiral, or partially achiral nature of the phosphorodithioate-containing oligonucleotides of the invention have the advantages described above including stability toward enzymatic and chemical hydrolysis as well as

other biochemical and biophysical properties desirable for *in vivo* antisense use including activation of RNase H.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature.

5

EXAMPLE 1

Synthesis of Ester Phosphoramidites

To 5'-O-4,4'-Dimethoxytrityl thymidine nucleoside (0.452 g, 0.830 mmol) in 5
10 jL of dry methylene chloride and 1 mL thiethylamine was added, followed by N,N
diisopropylphosphoramidic dichloride (0.2 g, 1 mmol) and the contents stirred at 0°C for
5 min. Then the ester alcohol 2 (0.16 g, 1 mmol) was added. The reaction mixture was
stirred at room temperature for 30 min. The solvent was then evaporated and the product
3 (0.5 g) was isolated following chromatography in silica gel
15 (hexane/CH₂Cl₂/EtOAc/N(Et)₃, 1/1/0.5/0.1).

EXAMPLE 2

Synthesis of thiaphospholidine synthons

20 To 25 g of racemic mixture of the aminothiols in tetrahydrofuran (500mL) and
triethyl amine (150 g) was added 13.8 g of PCI₃, at -78°C, over a period of 2 hours. After
the addition was over, the reaction mixture was allowed to warm to room temperature
over a period of 20 hours. The mixture was filtered under argon to give the crude
chlorophosphite, and is found to be of adequate purity for further reaction with the DMT-
25 T-nucleoside.

Approximately 5.4 g of 5'-O-dimethoxytrityl thymidine was dissolved in a
mixture of anhydrous ether (30 mL) and anhydrous triethylamine (6 mL). The solution
was gradually added to 3.1 g of the chlorophosphite at room temperature and the solution
stirred for 6 hours. The reaction mixture was poured into 200 mL of ice-cold water. it

was then extracted with ethyl acetate (3 x 200 mL). The organic layer was evaporated to dryness to give ca. 5g of the title compound as a pale yellow solid. Flash chromatographic purification (ethylacetate/methylene chloride/N(Et)₃, 80/19/1) on a silica gel column gives the title compound ca 3.8 g as pale yellow foam.

5

EXAMPLE 3

Synthesis of dinucleoside phosphorodithioates using a phosphorthioamidite synthon

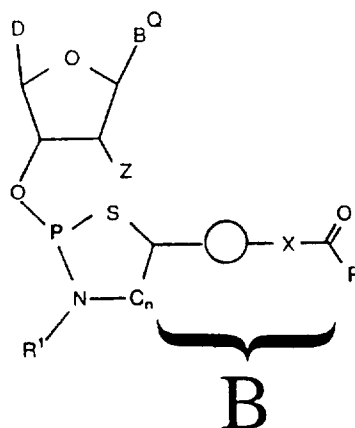
Having obtained the nucleoside phosphoramidite with the acyloxyaryl protecting group, the stage was set for its use in solid-phase coupling reaction. Thus contacting a solution of the midite in acetonitrile with a CPG-bound nucleoside (10 μ mol) for a period of two minutes in the presence of tetrazole as an activator followed by oxidation with 3H-benzodithiole-3-one-1, 1-dioxide (2% in acetonitrile) resulted in the formation of the dinucleoside phosphorodithioate triester with a coupling efficiency greater than 97% (as evaluated by trityl yields) (Iyer et al., *J. Am. Chem. Soc.*, vol. 112:1253-54 (1990), and Iyer et al., *J. Org. Chem.* vol. 55:4693-98 (1990). Following the synthesis, the CPG-bound product was heated with aqueous ammonium hydroxide at 55°C for 3 hours. Examination of the product revealed that the product as >96% the desired phosphorodithioate with the remaining approximately 4% material being the unreacted T nucleoside. The presence of phosphoromonothiate was less than 2% under these conditions.

What is claimed is:

1. A nucleoside synthon comprising a sulfur protecting group, said nucleoside synthon having the general structure:

5

10



15

20

25

wherein \bigcirc is aryl, heterocyclic, heteroaryl, polyaromatic, cycloalkenyl, alkenyl, alkynyl; R is aryl, heterocyclic heteroaryl, polyaromatic, alkenyl and substituted derivatives thereof, alkynyl or combinations of alkenyl moieties and alkynyl moieties; X is S, NH or O; n=1 or 0; R' is alkyl (1-15 carbons straight or branched), an aromatic or heterocyclic 5 or 6 membered ring comprising 1-5 heteroatoms, aralkyl or heteroaryl; B is a nucleoside base, D is a 5'OH blocking group, Q is a base-labile protecting group, capable of protecting exocyclic amino groups; Z is H, OH, an O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, an -O-aryl or O-allyl group having 2-6 carbons atoms wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy caroxyl, carbalkoxyl or amino and the sulfur protecting group is represented as B.

2. The nucleoside synthon of claim 1 wherein \bigcirc is a phenyl group, cis or trans ethenyl, or thiozole and R is isopropyl phenyl, cis or trans ethenyl, thiozole, pyrrole or furan.

3. The nucleoside synthon of claim 1 wherein Q is the base protecting group, N-pent-4-enoyl.

4. The nucleoside synthon of claim 1 wherein D is dimethoxytrityl or trityl.

5

5. A method for synthesizing a phosphorodithioate oligonucleotide, the method comprising the steps of coupling together two nucleoside synthons according to claim 1, sulfurizing the resulting intermediate to yield the protected phosphorodithioate oligonucleotide; chemoselectively removing the sulfur-protecting ester moiety to yield the phosphorodithioate.

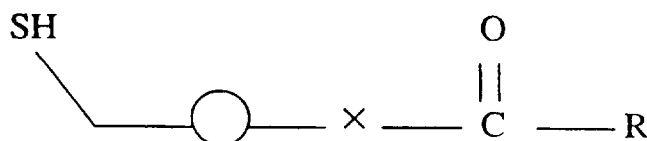
10

6. A method for synthesizing a phosphorodithioate oligonucleotide the method comprising the steps of coupling a synthon of claim 1 to a nucleoside that is covalently bound to a suitable solid support; sulfurizing the resulting intermediate to yield the protected phosphorodithioate oligonucleotide; and chemoselectively removing the sulfur-protecting ester moiety to yield the phosphorodithioate.

15

7. A method for synthesizing a phosphorodithioate oligonucleotide comprising the steps of: providing a thio-H-phosphonate nucleoside synthon; coupling in the presence of carbon tetrachloride, said synthon with a protected thiol moiety of the structure:

20

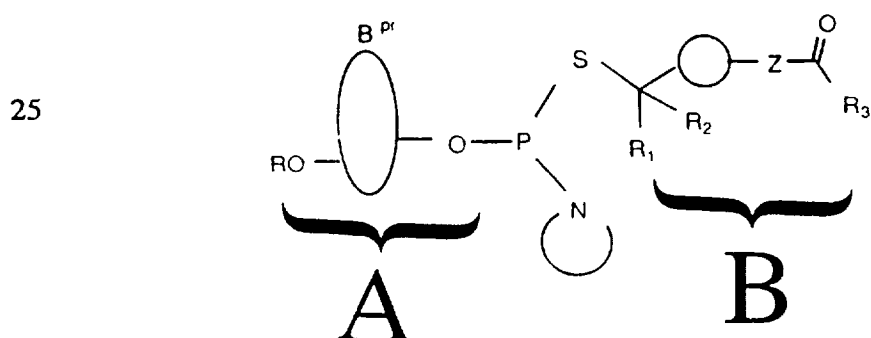


25

wherein \bigcirc is aryl, heterocyclic, heteroaryl, polyaromatic, cycloalkenyl, alkenyl, alkynyl; R is aryl, heterocyclic heteroaryl, polyaromatic, alkenyl and substituted derivatives thereof, alkynyl or combinations of alkenyl moieties and alkynyl moieties; X

is S, NH or O, to yield a sulphur protected thio-H-phosphonate intermediate and coupling said intermediate with a second thirH-phosphonate synthon.

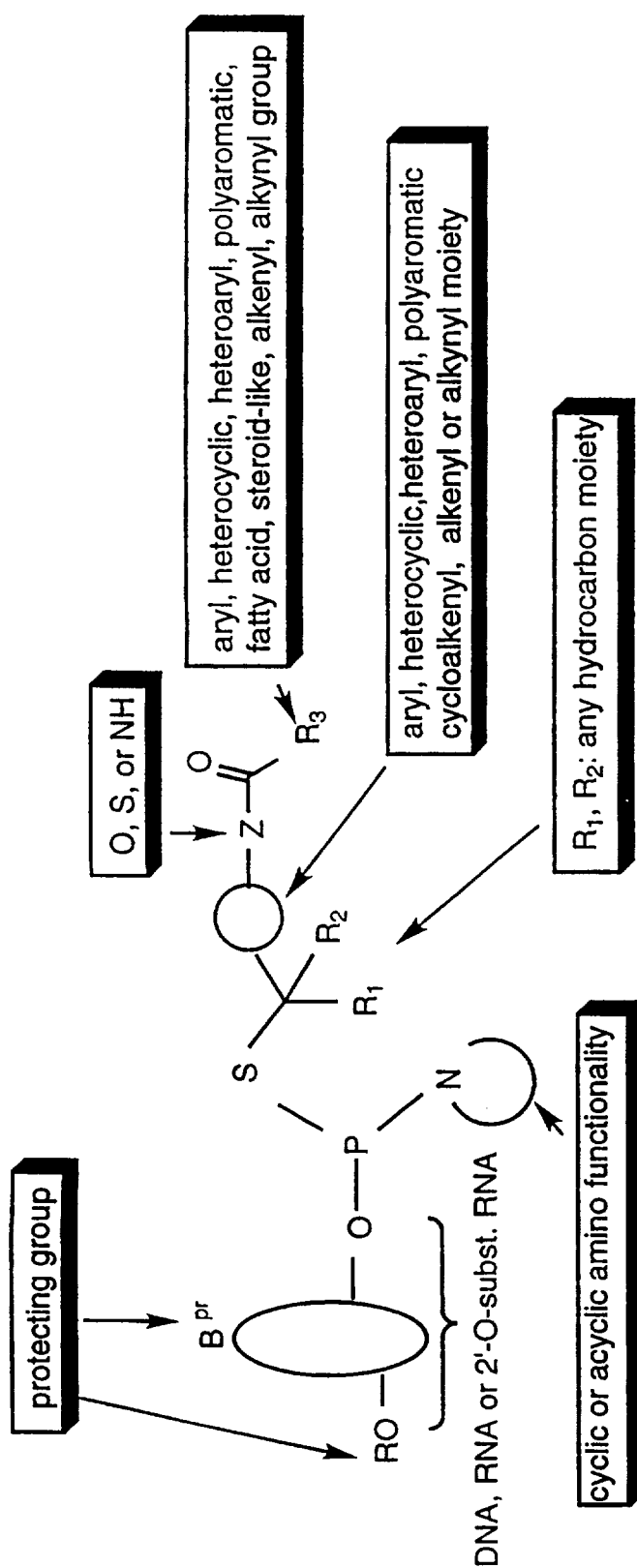
8. A population of phosphorodithioate-containing oligonucleotides with less than
5 2% contaminating phosphorothioate moieties incorporated therein.
9. A population of phosphorodithioate-containing mixed backbone oligonucleotides with less than 2% contaminating phosphorothioate moieties incorporated therein.
- 10 10. The oligonucleotids of claim 9 wherein each of said oligonucleotides comprise a phosphorodithioate region flanked on either side by a P-chiral methyl phosphonate region.
11. The oligonucleotides of claim 9 wherein each of said oligonucleotides comprises
15 a phosphodiester region flanked on either side by a phosphorodithioate region.
12. The oligonucleotides of claim 9 wherein each of said oligonucleotides comprises a P-chiral methylphosphonate retion flanked on either said by a phosphorodithioate region.
- 20 13. A nucleoside synthon comprising a sulfur protecting group, said nucleoside synthon having the general structure:



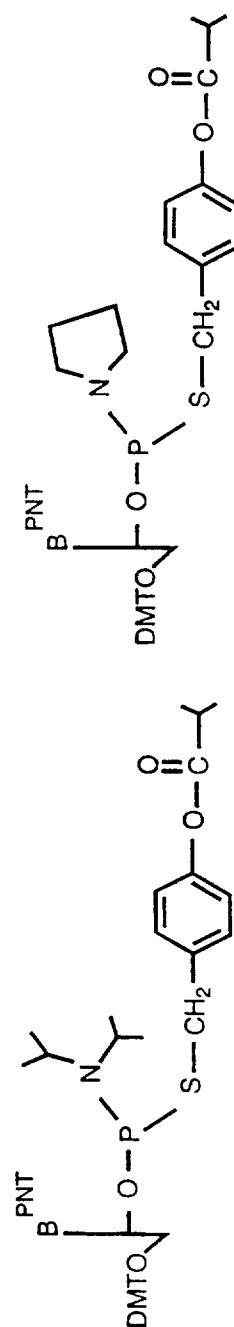
wherein \bigcirc is aryl, heterocyclic, heteroaryl, polyaromatic, cycloalkenyl, alkenyl, alkynyl; R_3 is aryl, heterocyclic heteroaryl, polyaromatic, alkenyl and substituted derivatives thereof, alkynyl or combinations of alkenyl moieties and alkynyl moieties; X is S, NH or O; R_1 and R_2 may be the same or different hydrocarbon moiety; R is a 5'OH blocking group, preferably dimethoxytrityl or trityl; B is a nucleoside base; P^r is a base protecting group, such as any suitable base labile group as is known in the art for the protection of exocyclic amino groups such as benzoyl groups or isobutyryl groups; A represents a nucleoside moiety which is a deoxyribonucleoside, a ribonucleoside, a substituted ribonucleoside wherein the 2' position of the ribose moiety is substituted with an O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an O-aryl or O-allyl group having 2-6 carbons atoms wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, e.g., with halo hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy carboxyl, carbalkoxyl or amino groups; \bigcirc^N represents a cyclic or acyclic functionality; and B represents the sulfur protecting group.

15

1/4

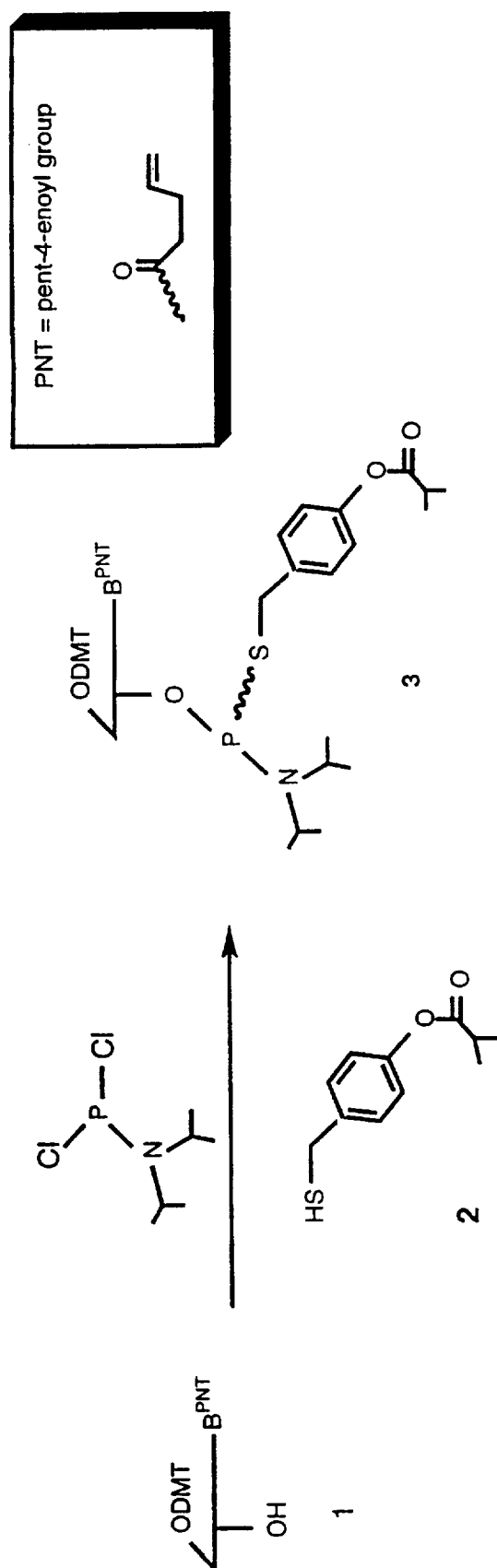
Fig. 1

Specific example of a phosphorothioamidite synthon



2/4

Fig. 2



3/4

Fig. 3

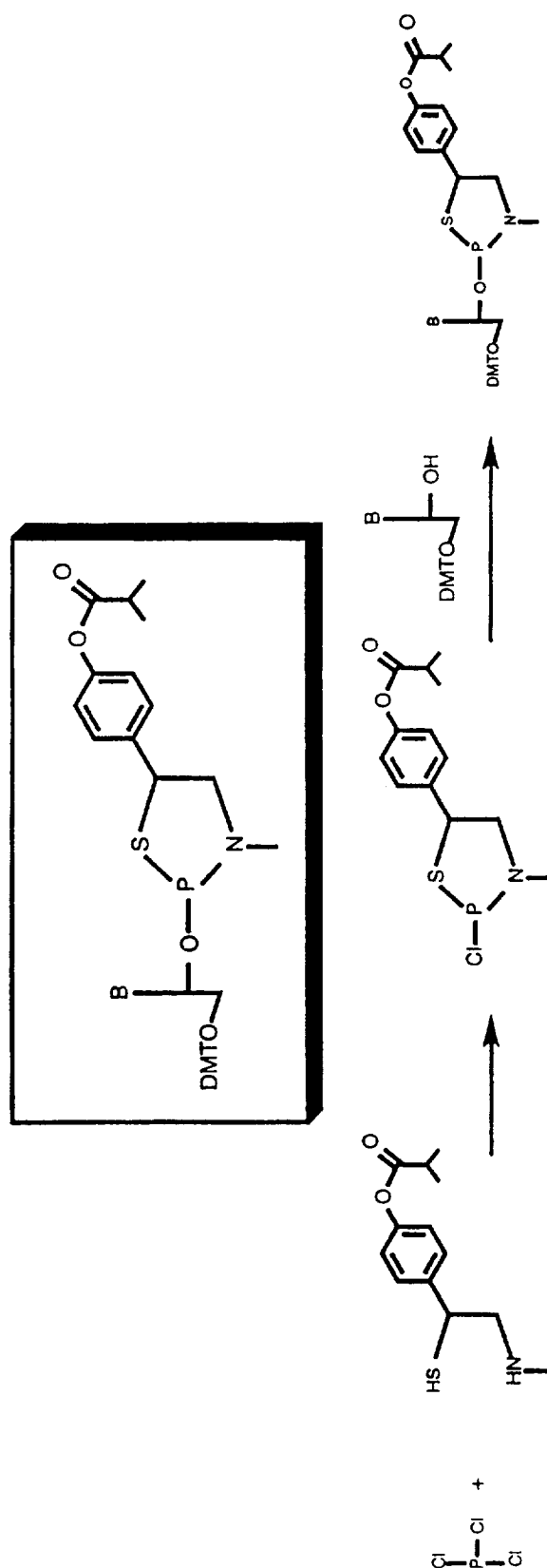
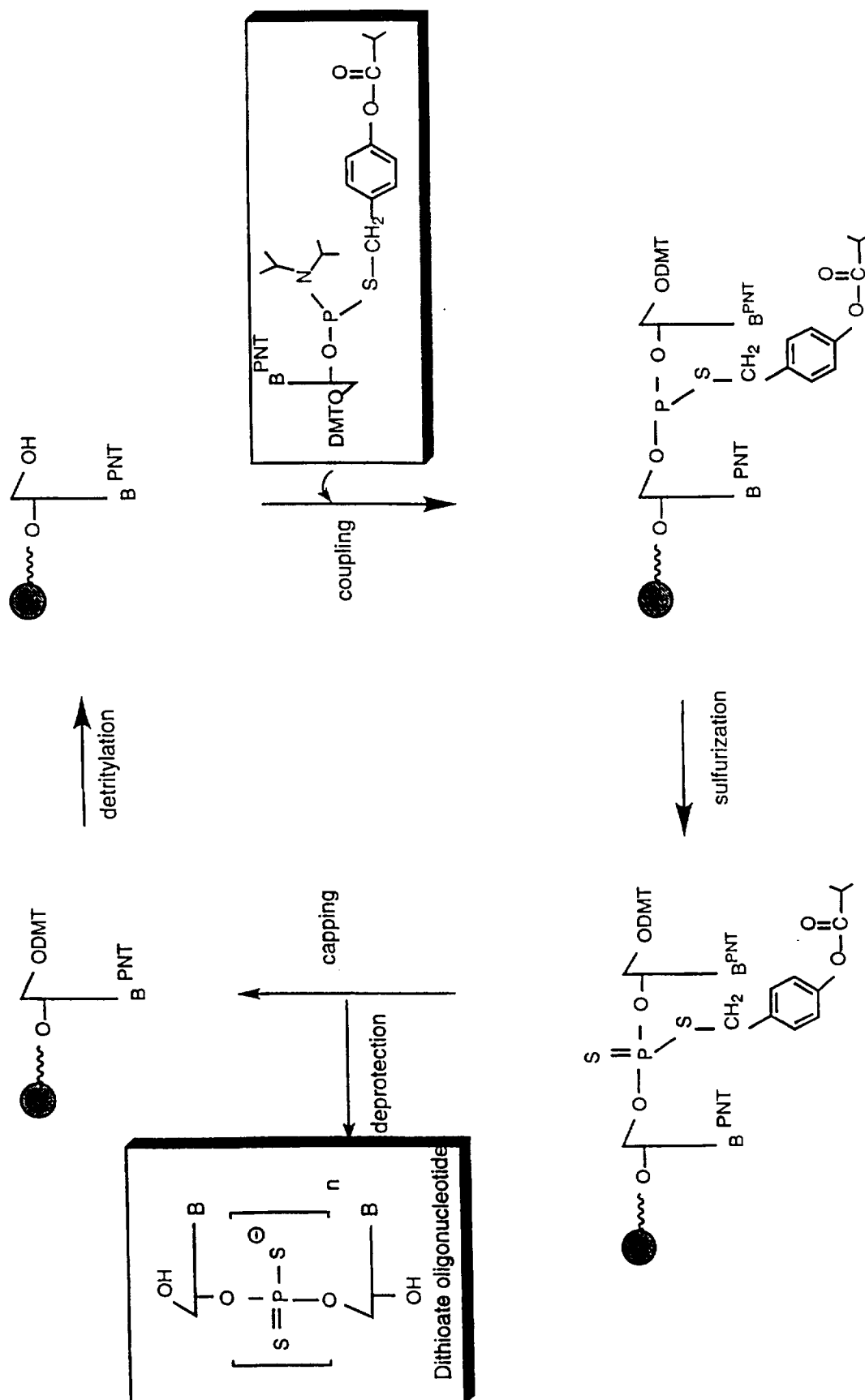


Fig. 4



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/14962

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07H21/00 C07H19/10 C07H19/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07H

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 07392 A (HYBRIDON INC ; IYER RADHAKRISHNAN P (US); YU DONG (US); AGRAWAL SUD) 14 March 1996	
A	--- RICHTER, WOLFGANG; KARL, ROSMARIE; UGI, IVAR: " A programmed five-membered cyclic phosphorylating reagent for the synthesis of oligonucleotides and its use" TETRAHEDRON, vol. 46, 1990, pages 3167-72, XP002048792	
X	--- S. AGRAWAL (ED.); W.T. WIESLER ET AL.: "Synthesis and Purification of Phosphorodithioate DNA" 1993 , HUMANA PRESS , TOTOWA XP002048793 chapter 9 see page 191 - page 206 -----	8-12

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

2 December 1997

Date of mailing of the international search report

16.12.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Bardili, W

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/14962

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9607392 A	14-03-96	AU 3675195 A	27-03-96
		CA 2199464 A	14-03-96
		EP 0779893 A	25-06-97
