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(54) Title: NOVEL SULFUR TRANSFER REAGENTS FOR OLIGONUCLEOTIDE SYNTHESIS**(57) Abstract**

In our research, two commercially available compounds, 3-amino-1,2,4-dithiazole-5-thione (1) and xanthane hydride (2), and their derivatives 3-6 are found to be potential sulfur-transfer reagents. The efficiency and optimization of these new sulfur-transfer reagents were investigated by solid-phase synthesis of dinucleotide and oligonucleotide phosphorothioates. The results show that both compounds (1) and (2) are highly efficiency sulfurizing reagents, and better than 99 % sulfur transfer efficiency can be achieved at each step. In contrast to Beaucage reagent, these novel sulfur-transfer reagents are very stable in various solvents, and are available in bulk quantities at low cost. Due to these advantages, compounds (1) and (2) can be considered alternatives to Beaucage reagent, especially in large-scale preparation of oligonucleotide phosphorothioates. Furthermore, compounds (1) and (2) were modified to enhance their solubility in acetonitrile. Three types of structure, as described as Formulae (I), (II) and (III), are considered as potential efficient sulfur-transfer reagents based on our results.

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**NOVEL SULFUR TRANSFER REAGENTS
FOR OLIGONUCLEOTIDE SYNTHESIS**

5 **Field of the Invention**

The invention relates to the chemical synthesis of oligonucleotides and to chemical entities useful in such synthesis. More particularly, the invention relates to sulfurization of the internucleoside linkages of oligonucleotides.

10

Summary of the Related Art

Oligonucleotides have become indispensable tools in modern molecular biology, being used in a wide variety of techniques, ranging from diagnostic 15 probing methods to PCR to antisense inhibition of gene expression.

Oligonucleotide phosphorothioates are of considerable interest in nucleic acid research and are among the analogues tested as oligonucleotide therapeutics.

20 Oligonucleotides phosphorothioates contain internucleotide linkages in which one of the nonbridging oxygen atoms of the phosphate group is replaced by a sulfur atom. This widespread use of oligonucleotides has led to an increasing demand for rapid, inexpensive, and efficient methods for synthesizing 25 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 30 (S. Agrawal, ed., Humana Press, 1993); *Oligonucleotides and Analogs: A Practical Approach*, pp. 87-108 (F. Eckstein, ed., 1991); and Uhlmann and Peyman, *Chemical Reviews*, 90: 543-584 (1990); Agrawal and Iyer, *Curr. Op. in*

Biotech. 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.* 72: 209 (1972) discloses phosphodiester chemistry for oligonucleotide synthesis. Reese, *Tetrahedron Lett.* 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

5 H-phosphonate approaches to automated synthesis. Beaucage and Carruthers, *Tetrahedron Lett.* 22: 1859-1862 (1981), discloses the use of deoxynucleoside phosphoramidites in polynucleotide synthesis. Agrawal and Zamecnik, U.S. Patent No. 5,149,798 (1992), discloses optimized synthesis of oligonucleotides

10 by the H-phosphonate approach.

These latter approaches have been used to synthesize oligonucleotides having a variety of modified internucleotide linkages. Agrawal and Goodchild, *Tetrahedron Lett.* 28: 3539-3542 (1987), teaches synthesis of oligonucleotide methylphosphonates using phosphoramidite chemistry. Connolly et al., *Biochemistry* 23: 3443 (1984), discloses synthesis of oligonucleotide phosphorothioates using phosphoramidite chemistry. Jager et al., *Biochemistry* 27: 7237 (1988), discloses synthesis of oligonucleotide phosphoramidates using phosphoramidite chemistry. Agrawal et al., *Proc. Natl. Acad. Sci. USA* 85: 7079-7083 (1988), discloses synthesis of oligonucleotide phosphoramidates and phosphorothioates using H-phosphonate chemistry.

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30 Solid phase synthesis of oligonucleotides by each of the foregoing processes involves the same generalized protocol. Briefly, this approach comprises anchoring the 3'-most nucleoside to a solid support functionalized

with amino and/or hydroxyl moieties and subsequently adding the additional nucleosides in stepwise fashion. Internucleoside linkages are formed between the 3' functional group of the incoming nucleoside and the 5' hydroxyl group 5 of the 5'-most nucleoside of the nascent, support-bound oligonucleotide. In the phosphoramidite approach, the internucleoside linkage is a phosphite linkage, whereas in the H-phosphonate approach, it is an H-phosphonate internucleoside linkage. To create the sulfur-containing phosphorothioate 10 internucleoside linkage, the phosphite or H-phosphonate linkage must be oxidized by an appropriate sulfur transfer reagent. In the H-phosphonate approach, this sulfurization is carried out on all of the H-phosphonate linkages 15 in a single step following the completion of oligonucleotide chain assembly, typically using elemental sulfur in a mixed solvent, such as CS₂/pyridine. In contrast, the phosphoramidite approach allows stepwise sulfurization to take place after each coupling, thereby providing the capability to control the state 20 of each linkage in a site-specific manner. Based on superior coupling efficiency, as well as the capacity to control the state of each linkage in a site-specific manner, the phosphoramidite approach appears to offer advantages.

Refinement of methodologies is still required, however, particularly 25 when making a transition to large-scale synthesis (10 μ mol to 1 mmol and higher). See Padmapriya et al., *Antisense Res. Dev.* 4: 185 (1994). Several modifications of the standard phosphoramidite processes have already been reported to facilitate the synthesis (Padmapriya et al., *supra*; Ravikumar et al., 30 *Tetrahedron* 50: 9255 (1994); Theisen et al., *Nucleosides & Nucleotides* 12: 43 (1994); and Iyer et al., *Nucleosides & Nucleotides* 14: 1349 (1995)) and isolation

(Kuijpers et al., *Nucl. Acids Res.* **18**: 5197 (1990); and Reddy et al., *Tetrahedron Lett.* **35**: 4311 (1994)) of oligonucleotides.

It is imperative that an efficient sulfur transfer reagent is used for the synthesis of oligonucleotide phosphorothioates via the phosphoroamidite approach. Elemental sulfur is not efficient due to poor solubility and slow sulfurization reaction. A number of more efficient sulfurizing reagents have been reported in recent years. These include phenylacetyl disulfide, (Kamer et al., *Tetrahedron Lett.* **30**: 6757-6760 (1989)), H-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent)(Iyer et al., *J. Org. Chem.* **55**: 4693-4699 (1990)), tetraethylthiuram disulfide (TETD)(Vu et al., *Tetrahedron Lett.* **32**: 3005-3008 (1991)), dibenzoyl tetrasulfide (Rao et al., *Tetrahedron Lett.* **33**: 4839-4842 (1992)), bis(O,O-diisopropoxyphosphinothiyl) disulfide (S-Tetra)(Stec et al., *Tetrahedron Lett.* **33**: 5317-5320 (1993)), benzyltriethyl-ammonium tetrathiomolybdate (BTM) (Rao et al., *Tetrahedron Lett.* **35**: 6741-6744 (1994)), bis(p-toluenesulfonyl) disulfide (Effimov et al., *Nucl. Acids Res.* **23**: 4029-4033 (1995)), 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH)(Xu et al., *Nucleic Acid Res.* **24**: 1602-1607 (1996)), and 1,2,4-dithiazolidine-3,5-dione (DtsNH)(Xu et al., *Nucleic Acid Res.* **24**: 1602-1607 (1996)). Both Beaucage reagent and TETD are commercially available.

Beaucage reagent has been widely used, however, its synthesis and stability are not optimal. In addition, the by-product formed by Beaucage reagent during sulfurization, 3H-2,1-benzoxanthiolan-3-one-1-oxide, is a potential oxidizing agent that can lead to undesired phosphodiester linkages under certain conditions. Therefore, its application in large-scale synthesis of oligonucleotide phosphorothioates may not be particularly suitable. We report two commercially available compounds **1** and **2** and novel analogues thereof

as potential alternative sulfurizing reagents.

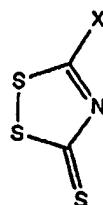
There is, therefore, a continuing need to develop new sulfur transfer reagents and processes for sulfurizing oligonucleotides. Ideally, such sulfur transfer reagents should be inexpensive to make, stable in storage, and highly efficient.

BRIEF SUMMARY OF THE INVENTION

10 The invention provides new processes for sulfur transfer reagents in sulfurizing oligonucleotides. The processes according to the invention yield inexpensive to make, stable in storage, and highly efficient in sulfurization.

15 In a first aspect, the invention provides novel sulfur transfer reagents having the structure according to Formula I:

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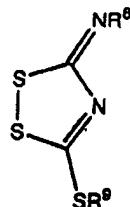


Formula I

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wherein X is R¹, NR²R³, NR⁴COR⁵, SR⁶, OR⁷; each R¹, R², R³, R⁴, and R⁵, is independently H or an alkyl or aromatic organic group; and each R⁶ and R⁷ is independently an alkyl or aromatic organic group.

In a second aspect, the invention relates to novel sulfur transfer reagents having the general structure according to Formula II:



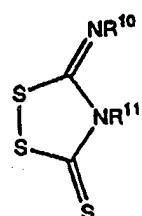
Formula II

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wherein R⁸ is H or an organic group; and R⁹ is an organic group.

Another aspect of the invention provides for novel sulfur transfer reagents having the general structure according to Formula III:

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Formula III

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wherein R¹⁰ and R¹¹ are each independently H or an organic group.

In a fourth aspect, the invention provides novel processes for adding a sulfur group to an internucleoside linkage of an oligonucleotide using the novel sulfur transfer reagents according to the invention. In preferred embodiments, the novel processes according to the invention comprise contacting an oligonucleotide having at least one sulfurizable internucleoside linkage with a novel sulfur transfer reagent according to the invention for a time sufficient for sulfurization of the sulfurizable internucleoside linkage(s) to occur.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the HPLC of the standard DMT protected T-T phosphothioate dimer. Figure 1A shows the HPLC of the standard P=S dimer.

5 Figure 1B shows the coinjection of P=S and P=O dimers.

Figure 2 shows the HPLC of DMT protected T-T phosphothioate dimer synthesized with compound 1 as a sulfur transfer reagent. Figure 2A represents sulfurization conditions with 4 equiv, 1 min. Figure 2B represents

10 10 sulfurization conditions with 4 equiv, 5 min. Figure 2C represents sulfurization conditions with 12 equiv, 1 min. Figure 2D represents sulfurization conditions with 12 equiv, 5 min.

Figure 3 is the HPLC of DMT protected T-T phosphothioate dimer

15 15 prepared with compound 2 as a sulfur transfer reagent.

Figure 4 is the HPLC of DMT protected T-T phosphothioate dimer prepared using compound 3 as a sulfur transfer reagent.

20 20 Figure 5 is the HPLC of DMT protected T-T phosphothioate dimer synthesized with compound 4 as a sulfur transfer reagent.

Figure 6 shows the HPLC of SEQ ID NO:1 synthesized with compound 1 as a sulfur transfer reagent. Figure 6A represents 1 min of sulfurization in

25 25 each synthetic cycle. Figure 6B represents 2 min of sulfurization in each synthetic cycle.

Figure 7 represents the CE of SEQ ID NO:1 synthesized with compound 1 as a sulfur transfer reagent, where the sulfurization occurred for 1 min.

30 30 Figure 8 represents the CE of SEQ ID NO: 1 synthesized with compound 1 as a sulfur transfer reagent, where the sulfurization occurred for 2 min.

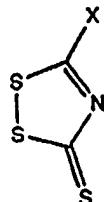
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to the chemical synthesis of oligonucleotides and 5 to chemical entities useful in such synthesis. More particularly, the invention relates to sulfurization of the internucleoside linkages of oligonucleotides. The 10 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 sulfur transfer reagents and processes for 15 their use in sulfurizing oligonucleotides. The sulfur transfer reagents according to the invention are inexpensive to make, stable in storage, and highly efficient 20 in sulfurization.

In a first aspect, the invention provides novel sulfur transfer reagents having the structure according to Formula I:

20



Formula I

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wherein X is R^1 , NR^2R^3 , NR^4COR^5 , SR^6 , OR^7 ; each R^1 , R^2 , R^3 , R^4 , and R^5 , is independently H or an alkyl or aromatic organic group; and each R^6 and R^7 is independently an alkyl or aromatic organic group.

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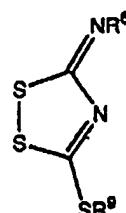
In a preferred embodiment, the sulfur transfer reagent having Formula I is 3-amino-1,2,4-dithiazole-5-thione (1), xanthane hydride (2), 3-N-acetyl-3-amino-1,2,4-dithiazole-5-thione (3), 3-N-trimethylacetyl-3-amino-1,2,4-dithiazole-

5-thione (4), 3-N-benzoyl-3-amino-1,2,4-dithiazole-5-thione (5), or 3-N-benzenesulfonyl-3-amino-1,2,4-dithiazole-5-thione (6).

In a second aspect, the invention relates to novel sulfur transfer reagents

5 having the general structure according to Formula II:

10



Formula II

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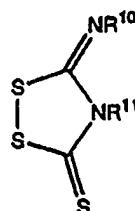
wherein R⁸ is H or an organic group; and R⁹ is an organic group.

Another aspect of the invention provides for novel sulfur transfer

15

reagents having the general structure according to Formula III:

20



Formula III

wherein each R¹⁰ and R¹¹ is independently H or an organic group.

25

In a preferred embodiment, the sulfur transfer reagent having Formula III is 3-amino-1,2,4-dithiazole-5-thione (1), xanthane hydride (2), 3-N-acetyl-3-amino-1,2,4-dithiazole-5-thione (3), 3-N-trimethylacetyl-3-amino-1,2,4-dithiazole-5-thione (4), 3-N-benzoyl-3-amino-1,2,4-dithiazole-5-thione (5), or 3-N-benzenesulfonyl-3-amino-1,2,4-dithiazole-5-thione (6).

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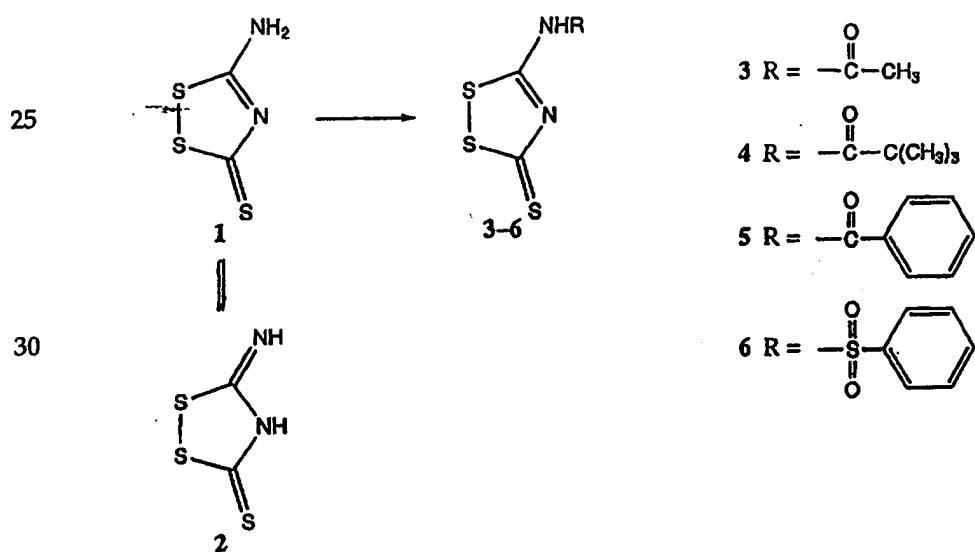
The compounds 1 and 2, 3-amino-1,2,4-dithiazole-5-thione and xanthane hydride respectively, are commercially available from several chemical

companies, including Lancaster, Crescent Chemicals, Maybridge, and TCI America. The purity of compounds **1** and **2** obtained is higher than 98%, and both compounds can be directly used in oligonucleotide synthesis without any 5 additional purification. These compounds have been used in the rubber industry as vulcanization reagents. However, these compounds, to our best knowledge, have not been applied in the synthesis of oligonucleotides.

Derivatives of compound **1** may be easily synthesized. Starting from 10 compound **1**, 3-N-acetyl-3-amino-1,2,4-dithiazole-5-thione (**3**), 3-N-trimethylacetyl-3-amino-1,2,4-dithiazole-5-thione (**4**), 3-N-benzoyl-3-amino-1,2,4-dithiazole-5-thione (**5**), and 3-N-benzenesulfonyl-3-amino-1,2,4-dithiazole-5-thione (**6**) were conveniently prepared by solid-phase phosphoramidite 15 approach in a better than 70% yield according to Scheme 1.

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Scheme 1



As explained in greater detail below, the reaction conditions included acetic anhydride/pyridine for 3 (96%); trimethyl acetic anhydride/Et₃N for 4 (70%); benzoyl chloride/Et₃N for 5 (70%); and benzene sulfonyl chloride/Et₃N for 6 (76%). The final products can be purified by a simple precipitation.

5 Stability and solubility studies of the sulfur transfer reagents were performed. Compounds 1-6 are stable in CH₃CN, pyridine-CH₃CN (1:9), and pyridine for more than one week. No precipitation occurred during testing 10 period. All the testing reagents are stable during the operation of automatic DNA synthesizer, no clogging or other problems were observed during the syntheses.

15 Parent compounds 1 and 2 can be dissolved in CH₃CN to form a 0.01 M solution. Their solubility can be increased with addition of pyridine [0.02 M in pyridine-CH₃CN (1:9); 0.5 M in neat pyridine]. Derivatization of the parent compounds also can improve the solubility in CH₃CN (>0.02 M), e.g., 20 compound 4 has a solubility better than 1 M in neat CH₃CN.

Accordingly, it has been demonstrated that the oligonucleotide phosphorothioates can be efficiently prepared by solid-phase phosphoramidite 25 approach using 3-amino-1,2,4-dithiazole-5-thione (1), xanthane hydride (2), and their corresponding derivatives. Compounds 1 and 2 are commercially available and relatively inexpensive compared with currently used sulfurizing reagents, such as Beaucage reagent and EDITH. Due to its high efficiency and 30 low cost, compounds 1 and 2, and their appropriately modified analogues can be considered as an advantageous alternative to Beaucage reagent, especially in large-scale preparation of oligonucleotide phosphorothioates.

In another aspect, the invention provides novel processes for adding a sulfur group to an internucleoside linkage of an oligonucleotide using the novel sulfur transfer reagents according to the invention. In preferred 5 embodiments, the novel processes according to the invention comprise contacting an oligonucleotide having at least one sulfurizable internucleoside linkage with a novel sulfur transfer reagent according to the invention for a time sufficient for sulfurization of the sulfurizable internucleoside linkage(s) to 10 occur. Each sulfurizable internucleoside linkage preferably contains a phosphorous (III) atom. In a particularly preferred embodiment, the sulfurizable internucleoside linkage is a phosphite, thiophosphite, H-phosphonate, thio-H-phosphonate, or alkylphosphite (especially 15 methylphosphite) internucleoside linkage. Preferably, the sulfurization reaction would be allowed to proceed to a sulfur transfer efficiency greater than that expected for the prior art compounds, as measured by ^{31}P -NMR. In typical 20 synthesis conditions such efficiency is achieved within from about 1 to about 5 minutes reaction time with the novel transfer reagents. Typically, the reaction takes place in pyridine, THF, or mixtures thereof. For purposes of this aspect of the invention, the term oligonucleotide includes linear polymers of two or 25 more natural deoxyribonucleotide, ribonucleotide, or 2'-O-substituted ribonucleotide monomers, or any combination thereof. The term oligonucleotide also encompasses such polymers having chemically modified 30 bases or sugars and/or non-nucleosidic analogs linked by phosphodiester bonds or analogs thereof ranging in size from a few monomeric units, e.g., 2-3, to several hundred monomeric units and/or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines

and adamantane. In particular, oligonucleotides may also include non-natural oligomers having phosphorous-containing internucleoside linkages whose phosphorous (III) precursors are amenable to sulfurization, (See, e.g., Takeshita et al., *J. Biol. Chem.* 282: 10171-10179 (1987).) 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 halogen, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxy, carbalkoxy, or amino groups; or with a hydroxy, an amino or a halogen group, but not with a 2'-H group. Such oligonucleotides may include any of the internucleoside linkages which are known in the art, including without limitation phosphorothioate, phosphorodithioate, alkylphosphonate (especially methylphosphonate), phosphoramidate, amide (PNA), carbamate, and alkylphosphonothioate linkages. In a preferred embodiment, the oligonucleotide is bound to a solid support, but such oligonucleotides may be sulfurized in solution phase as well.

The efficiency of these new sulfur-transfer reagents was first evaluated by solid-phase syntheses of dinucleotide phosphorothioate, 5'-DMT-TT-OH-3'. (Figure 1.) Synthesis was performed at 1.0 μ mol scale using the standard phosphorothioate protocol ("THIO 1 μ mol"). The dinucleotide phosphorothioate was analyzed by reverse-phase HPLC. The results show that a better than 99% sulfur transfer efficiency was achieved using compounds 1 and 2. (Figures 2 and 3, respectively.) Compounds 3-4 were also found to be efficient sulfur-transfer reagents. (Figures 4-5.)

To further evaluate the usefulness of compounds 1 and 2 as efficient sulfurizing reagents, an oligonucleotide phosphorothioate, 5'-CTCTCGCACCCATCTCTCTCCTTCT-3' (SEQ ID NO:1), was synthesized at 1.0 5 μ mol scale using a 0.02 M solution in CH₃CN/pyridine (9:1) for 1 or 2 min of sulfurization in each synthetic cycle. After ammonolytic release from CPG and deprotection, the crude oligonucleotide phosphorothioate was analyzed by ion exchange-HPLC and CE. (Figures 6-8.) The results show that more than 99.5% 10 sulfur transfer efficiency was achieved at each step, and a yield better than 80% has been achieved in these syntheses (see Figure 3).

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Examples

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in 20 nature.

In general, anhydrous pyridine and CH₂Cl₂ were purchased from Aldrich (Milwaukee, WI). Anhydrous CH₃CN was purchased from J. T. Baker Inc. (Phillipsburg, NJ). dT-CPG, 5'-DMT-thymidine cyanoethyl 25 phosphoramidite, Cap A, Cap B, activator, oxidizing and deblock solutions were purchased from PerSeptive Biosystems, (Framingham, MA). Beaucage reagent (³H-1,2-benzodithiol-3-one-1,1-dioxide) was purchased from R. I. Chemical (Orange, CA). All other chemicals were purchased from Aldrich. ³¹P NMR spectra (121.65 MHz) and ¹H NMR spectra (300 MHz) were recorded on 30 a Varian UNITY 300 (the chemical shift was correlated to 85% H₃PO₄ and tetramethylsilane, respectively). Dinucleotide and oligonucleotide syntheses

were performed on an automated nucleic acid synthesizer (8909 Expedite™, Millipore, Bedford, MA). Reverse phase HPLC was performed on a Waters 600E pump with a Waters 440 absorbance detector, Waters 746 integrator, and

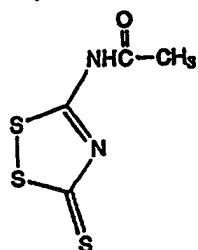
5 a Nova-Pak C18 (3.9 X 150 mm) column, using a linear gradient of CH₃CN-aqueous NH₄OAc (0.1 M) (4:1) and 0.1 M aqueous NH₄OAc from 1:9 to 3:2 over a period of 40 min, flow rate 2 mL/min, detection at 260 nm. Ion-exchange HPLC analyses were performed on a Beckman System Gold 126 with
10 1 a Beckman 166 absorbance detector on a NUCEOPAC PA-100 column (4 x 50 mm) using a linear gradient of Buffer A (25 mM of Tris-HCl and 1 mM EDTA, CH₃CN, pH=8) and Buffer B (2 M NaCl and buffer A) from 100% A to 100% B over a period of 5 min, then maintained at 100% B for 3 min, flow rate 2
15 mL/min, detection at 254 nm. Capillary electrophoresis was performed on a Beckman P/ACE System 5010. Samples were injected for 5 seconds and analyzed for 40 min.

20 The compound 3-amino-1,2,4-dithiazole-5-thione (1) was commercially available at 98% purity from several chemical companies, including Lancaster, Crescent Chemicals, and Maybridge, and can be directly used without further purification.

25 The compound xanthane hydride (2) was commercially available from TCI America at 98% purity, and can be directly used without any further purification.

Example 1**Synthesis of 3-N-acetyl-3-amino-1,2,4-dithiazole-5-thione (3)**

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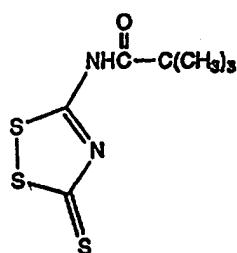


Neat acetic anhydride (1.49 mL, 16 mmol) was added dropwise to the
 10 suspension of 3-amino-1,2,4-dithiazole-5-thione (compound 1, 2.0 g, 13 mmol)
 in the mixture of pyridine-CH₃CN (1:5, 50 mL) over a period of 30 min. The
 resultant clear yellow solution was stirred at 25°C for 1 h, then concentrated
 15 under reduced pressure. The resultant yellow solid was dissolved in EtOAc
 (500 mL), washed with 10% aqueous citric acid (500 mL), H₂O (2 x 500 mL),
 then brine (500 mL), and then dried over Na₂SO₄. Evaporation of the organic
 solvent gave the title yellow solid. Yield: 2.50 g (96%); m.p. 229.6-230.1°C.

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Example 2**Synthesis of 3-N-trimethylacetyl-3-amino-1,2,4-dithiazole-5-thione (4)**

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Neat trimethylacetic anhydride (3.20 mL, 16 mmol) was added to the
 suspension of 3-amino-1,2,4-dithiazole-5-thione (compound 1, 2.0 g, 13 mmol)
 in the mixture of Et₃N-CH₂Cl₂ (1:5, 30 mL) in one portion. A clear yellow

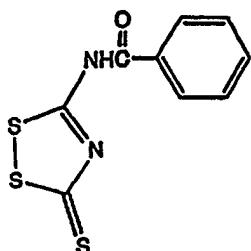
solution was obtained in 10 min, and stirred at 25°C for 2 h, and then concentrated under reduced pressure. The resultant yellow solid was dissolved in EtOAc (100 mL), washed with 10% aqueous citric acid (2 x 100 mL), H₂O (2 x 100 mL), then brine (100 mL), and then dried over Na₂SO₄.
5 Organic solvent was removed under reduced pressure, the resultant yellow solid was treated with hexane (100 mL) and stored at 4 °C for 2 h. The yellow precipitate was collected by filtration, washed with hexane (3 x 5 mL), and
10 dried *in vacuo*. Yield: 2.17 g (70%); m.p. 179.6-179.6°C.

Example 3

Synthesis of 3-N-benzoyl-3-amino-1,2,4-dithiazole-5-thione (5)

15

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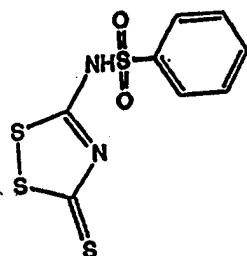
3-Amino-1,2,4-dithiazole-5-thione (compound 1, 1.0 g, 6.6 mmol) was suspended in the mixture of Et₃N-CH₂Cl₂ (1:5, 20 mL), followed by addition of
25 benzoic chloride (0.92 mL, 1.2 equiv). A clear yellow solution was obtained in 5 min. Reaction solution was stirred at 25°C for 2 h, then concentrated under reduced pressure to remove organic solvent. The resultant yellow solid was treated with EtOAc (200 mL), the insoluble solid was removed by filtration, and the EtOAc layer was washed with 10% aqueous citric acid (2 x 200 mL), H₂O (2 x 200 mL), brine (200 mL), and dried over Na₂SO₄. Evaporation of EtOAc gave a yellow solid, which was treated with CH₂Cl₂ (100 mL), then
30

followed by addition of hexane (100 mL). The resultant solid was removed by filtration, and the filtrate was removed under reduced pressure to give a yellow solid. Yield: 1.17 g (70%).

5

Example 4**Synthesis of 3-N-benzenesulfonyl-3-amino-1,2,4-dithiazole-5-thione (6)**

10



15

Except that benzene sulfonyl chloride was used instead of benzoic chloride, the reaction conditions and work-up process are identical to the preparation of compound 5. Yield: 1.46 g (76%).

20

Example 5**Stability and Solubility Studies of Xanthane Hydride, 3-Amino-1,2,4-dithiazole-5-thione, and Their Corresponding Derivatives in CH₃CN**

The solubility and stability studies were carried out in CH₃CN, pyridine-CH₃CN (1:9), and pyridine at 25°C in all the cases. The saturated solution was dried, and the solubility was determined by the weight of the testing compounds in the exact volume of testing solution. The stability was determined based on either TLC or HPLC analyses.

30

Example 6**Synthesis of Dinucleotide Phosphorothioate**

Dimer was assembled on a PerSeptive DNA/RNA (Millipore 8909

5 Expedite™, Millipore, Bedford, MA) on a 0.1 μ mol scale using the synthesis protocol "THIO 1 μ mol" (Expedite software version 1.01), starting from DMT-T-Su-CPG (500 Å, loading: 60 μ mol/g). Sulfurizing reagents were prepared in either CH₃CN or pyridine-CH₃CN (1:9) at a concentration of 0.02 M unless
10 specified. Sulfurization was carried out using 4 equiv or 12 equiv of sulfurizing reagents for 1 or 5 min reaction, respectively. Final cleavage was carried out by treatment using concentrated ammonium hydroxide (1 μ mol/1 mL) at 25°C for 1 h. CPG and other insoluble residues were removed by
15 filtration, and the ammonium hydroxide solution was dried by lyophilization. HPLC analysis was carried out using a linear gradient of 0.1 M aqueous NH₄OAc and CH₃CN-aqueous NH₄OAc (0.1 M) (4:1) from 1:9 to 3:2 over a
20 period of 40 min, flow rate 1.0 mL/min, detection at 260 nm. 5'-DMT-T(P=S)-T-OH-3' diastereomers were eluted at t_R = 31.6 min and t_R = 31.7 min. The corresponding P=O dimer was eluted at t_R = 28.4 min. (Figure 1.)

25

Example 7**Synthesis of a Oligonucleotide Phosphorothioate (5'-**

CTCTCGCACCCATCTCTCTCCTTCT-3' (SEQ ID NO:1))

The chain assembly was carried out using the same protocol as
30 described in the dinucleotide phosphorothioate synthesis. Sulfurizing reagent (compound 1) was prepared at a concentration of 0.02 M in pyridine-CH₃CN (1:9), and sulfurization was carried out using 12 equiv for 1 or 2 min,

respectively. Ion exchange HPLC and CE analyses were performed to evaluate the synthesis of this oligomer.

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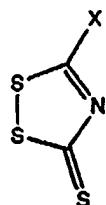
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What we claim:

1. A sulfur transfer reagent having the general structure according to Formula

5 I



Formula I

10

wherein X is R^1 , NR^2R^3 , NR^4COR^5 , SR^6 , OR^7 ; each R^1 , R^2 , R^3 , R^4 , and R^5 , is independently H or an alkyl or aromatic organic group.

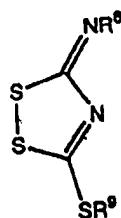
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2. The sulfur transfer reagent according to claim 1 wherein Formula I is 3-amino-1,2,4-dithiazole-5-thione, xanthane hydride, 3-N-acetyl-3-amino-1,2,4-dithiazole-5-thione, 3-N-trimethylacetyl-3-amino-1,2,4-dithiazole-5-thione, 3-N-benzoyl-3-amino-1,2,4-dithiazole-5-thione, or 3-N-benzenesulfonyl-3-amino-1,2,4-dithiazole-5-thione.

20

3. A sulfur transfer reagent having the general structure according to Formula

25 II



Formula II

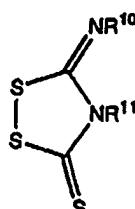
30

wherein R^8 is H or an organic group; and R^9 is an organic group.

4. A sulfur transfer reagent having the general structure according to Formula

III

5



Formula III

wherein R¹⁰ and R¹¹ are each independently H or an organic group.

10

5. The sulfur transfer reagent according to claim 4 wherein Formula III is
 Formula I is 3-amino-1,2,4-dithiazole-5-thione, xanthane hydride, 3-N-acetyl-3-
 amino-1,2,4-dithiazole-5-thione, 3-N-trimethylacetyl-3-amino-1,2,4-dithiazole-5-
 15 thione, 3-N-benzoyl-3-amino-1,2,4-dithiazole-5-thione, or 3-N-benzenesulfonyl-3-
 amino-1,2,4-dithiazole-5-thione.

20

6. A process for adding a sulfur group to an internucleoside linkage of an
 oligonucleotide, the process comprising contacting an oligonucleotide having at
 least one sulfurizable internucleoside linkage with the novel sulfur transfer
 reagent according to claim 1 for a time sufficient for sulfurization of the
 25 sulfurizable internucleoside linkage(s) to occur.

7. The process according to claim 6, wherein each sulfurizable internucleoside
 linkage contains a phosphorous (III) atom.

30

8. The process according to claim 7, wherein the sulfurizable internucleoside linkage is a phosphite, thiophosphite, H-phosphonate, thio-H-phosphonate, or alkylphosphite internucleoside linkage.

5

9. The process according to claim 8, wherein the sulfurization reaction is allowed to proceed from about 1 to about 5 minutes.

10

10. A process for adding a sulfur group to an internucleoside linkage of an oligonucleotide, the process comprising contacting an oligonucleotide having at least one sulfurizable internucleoside linkage with the novel sulfur transfer reagent according to claim 2 for a time sufficient for sulfurization of the 15 sulfurizable internucleoside linkage(s) to occur.

15

20

11. The process according to claim 10, wherein each sulfurizable internucleoside linkage contains a phosphorous (III) atom.

20

25

12. The process according to claim 11, wherein the sulfurizable internucleoside linkage is a phosphite, thiophosphite, H-phosphonate, thio-H-phosphonate, or alkylphosphite internucleoside linkage.

30

13. The process according to claim 12, wherein the sulfurization reaction is allowed to proceed from about 1 to about 5 minutes.

14. A process for adding a sulfur group to an internucleoside linkage of an oligonucleotide, the process comprising contacting an oligonucleotide having at

least one sulfurizable internucleoside linkage with the novel sulfur transfer reagent according to claim 3 for a time sufficient for sulfurization of the sulfurizable internucleoside linkage(s) to occur.

5

15. The process according to claim 14, wherein each sulfurizable internucleoside linkage contains a phosphorous (III) atom.

10

16. The process according to claim 15, wherein the sulfurizable internucleoside linkage is a phosphite, thiophosphite, H-phosphonate, thio-H-phosphonate, or alkylphosphite internucleoside linkage.

15

17. The process according to claim 16, wherein the sulfurization reaction is allowed to proceed from about 1 to about 5 minutes.

20

18. A process for adding a sulfur group to an internucleoside linkage of an oligonucleotide, the process comprising contacting an oligonucleotide having at least one sulfurizable internucleoside linkage with the novel sulfur transfer reagent according to claim 4 for a time sufficient for sulfurization of the

25

sulfurizable internucleoside linkage(s) to occur.

19. The process according to claim 18, wherein each sulfurizable internucleoside linkage contains a phosphorous (III) atom.

30

20. The process according to claim 19, wherein the sulfurizable internucleoside linkage is a phosphite, thiophosphite, H-phosphonate, thio-H-phosphonate, or alkylphosphite internucleoside linkage.

5

21. The process according to claim 20, wherein the sulfurization reaction is allowed to proceed from about 1 to about 5 minutes.

10

22. A process for adding a sulfur group to an internucleoside linkage of an oligonucleotide, the process comprising contacting an oligonucleotide having at least one sulfurizable internucleoside linkage with the novel sulfur transfer reagent according to claim 5 for a time sufficient for sulfurization of the 15 sulfurizable internucleoside linkage(s) to occur.

15

23. The process according to claim 22, wherein each sulfurizable internucleoside linkage contains a phosphorous (III) atom.

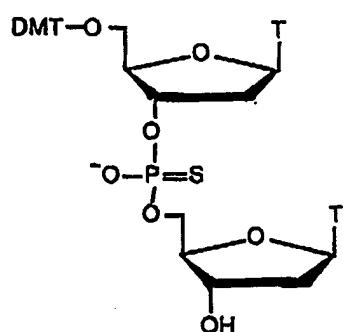
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24. The process according to claim 23, wherein the sulfurizable internucleoside linkage is a phosphite, thiophosphite, H-phosphonate, thio-H-phosphonate, or 25 alkylphosphite internucleoside linkage.

25. The process according to claim 24, wherein the sulfurization reaction is allowed to proceed from about 1 to about 5 minutes.

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(Standard)

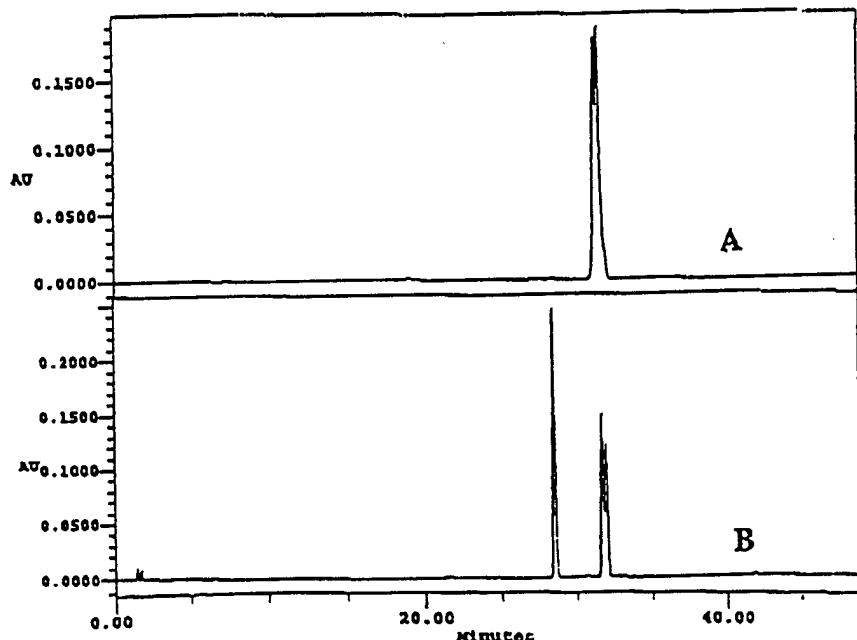


Figure 1. HPLC of standard DMT protected T-T phosphothioate dimer. HPLC was carried on a Nova-Pak C18 column (60 Å, 3.9 x 150 mm) using a linear gradient of 0.1 M aqueous NH₄OAc and CH₃CN-aqueous NH₄OAc (0.1 M) (4:1) from 1:9 to 3:2 over a period of 40 min, detection at 254 nm, flow rate 1.0 mL/min. A: standard P=S dimer; B: coinjection of P=S and P=O dimers.

Synthesis of 5'-DMT-T(P=S)-T-CM
Using 3-Amino-1,2,4-Dithiazole-5-Thione

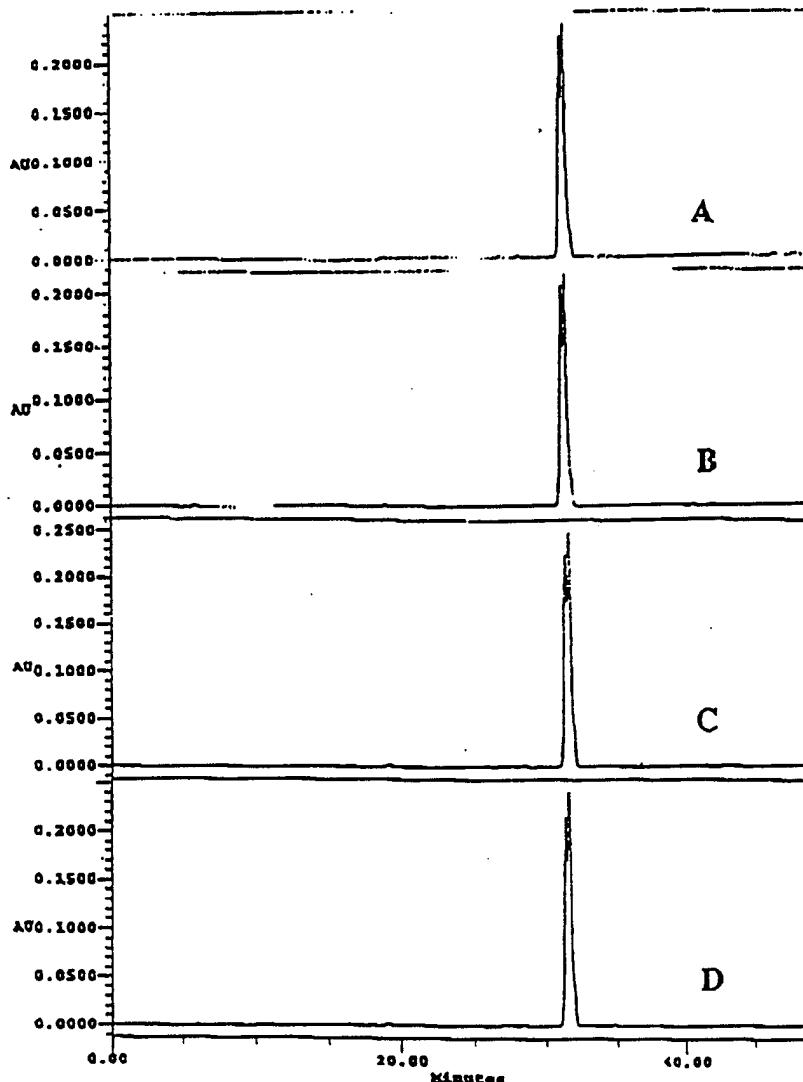


Figure 2. HPLC of DMT protected T-T phosphothioate dimer prepared using 3-amino-1,2,4-dithiazole-5-thione as sulfurizing reagent. HPLC conditions as Figure 1. Sulfurization was carried out at a concentration of 0.02 M in pyridine-CH₃CN (1:9): A: 4 equiv, 1 min (purity, 98.3%; P=O, 0.32%); B: 4 equiv, 5 min (purity, 98.5%; P=O, 0.28%); C: 12 equiv; 1 min (purity, 98.3%; P=O, 0.44%); D: 12 equiv, 5 min (purity, 98.5%; P=O, 0.38%).

Synthesis of 5'-DMT- T(P=S)-T-OH
Using Xanthane Hydride

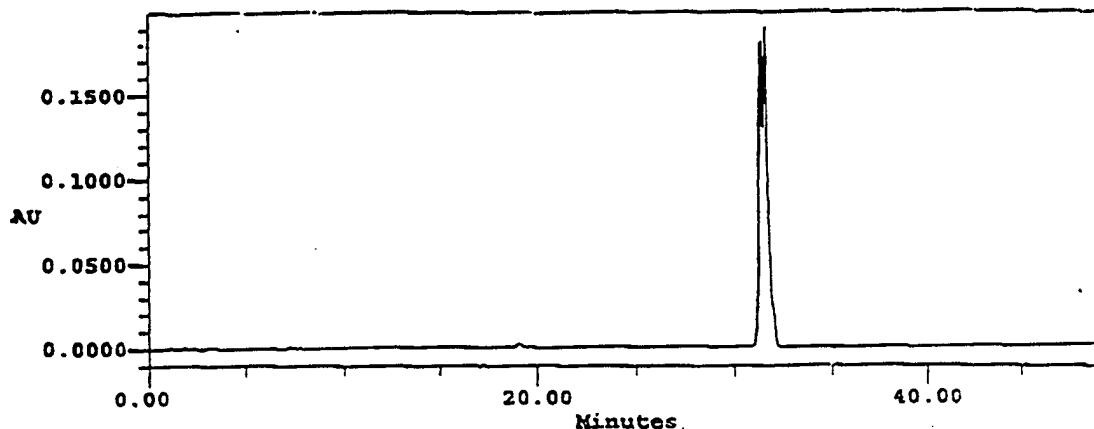


Figure 3. HPLC of DMT protected T-T phosphothioate dimer prepared using xanthane hydride as sulfurizing reagent. HPLC conditions as Figure 1. Sulfurization was carried out in pyridine-CH₃CN (1:9) at a concentration of 0.02 M (12 equiv) for 5 min. Purity: 98.5%; P=O: 0.35%.

Synthesis of 5'-DMT- T(P=S)-T-OH

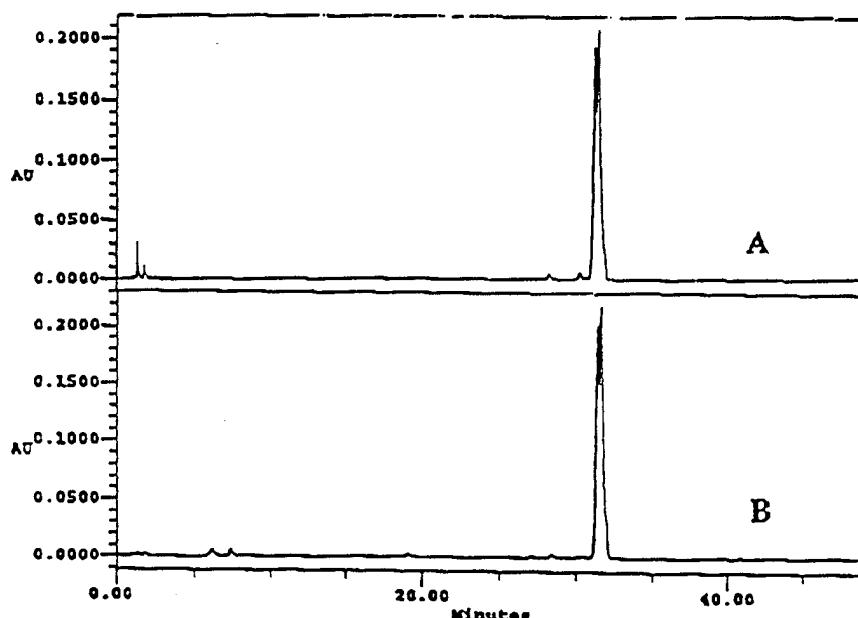


Figure 4. HPLC of DMT protected T-T phosphothioate dimer prepared using 3-N-acetyl-3-amino-1,2,4-dithiazole-5-thione as sulfurizing reagent. HPLC conditions as Figure 1. Sulfurization was carried out: A: 0.02 M (12 equiv) in pyridine-CH₃CN (1:9), 1 min (purity: 96.6%, P=O, 1.5%); B: 0.02 M (12 equiv) in CH₃CN, 5 min (purity: 96.6%, P=O, 1.5%).

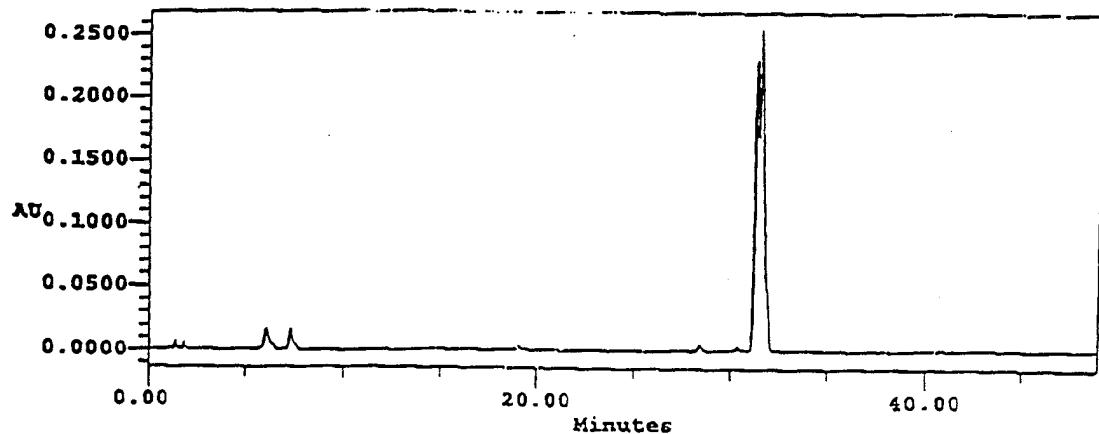
Synthesis of 5'-DMT- T(P=S)-T-OH

Figure 5. HPLC of DMT protected T-T phosphothioate dimer prepared using 3-*N*-trimethylacetyl-3-amino-1,2,4-dithiazole-5-thione as sulfurizing reagent. HPLC conditions same as Figure 1. Sulfurization was carried out in CH₃CN at a concentration of 0.06 M (12 equiv) for 5 min (purity: 90.7%; 1.09%).

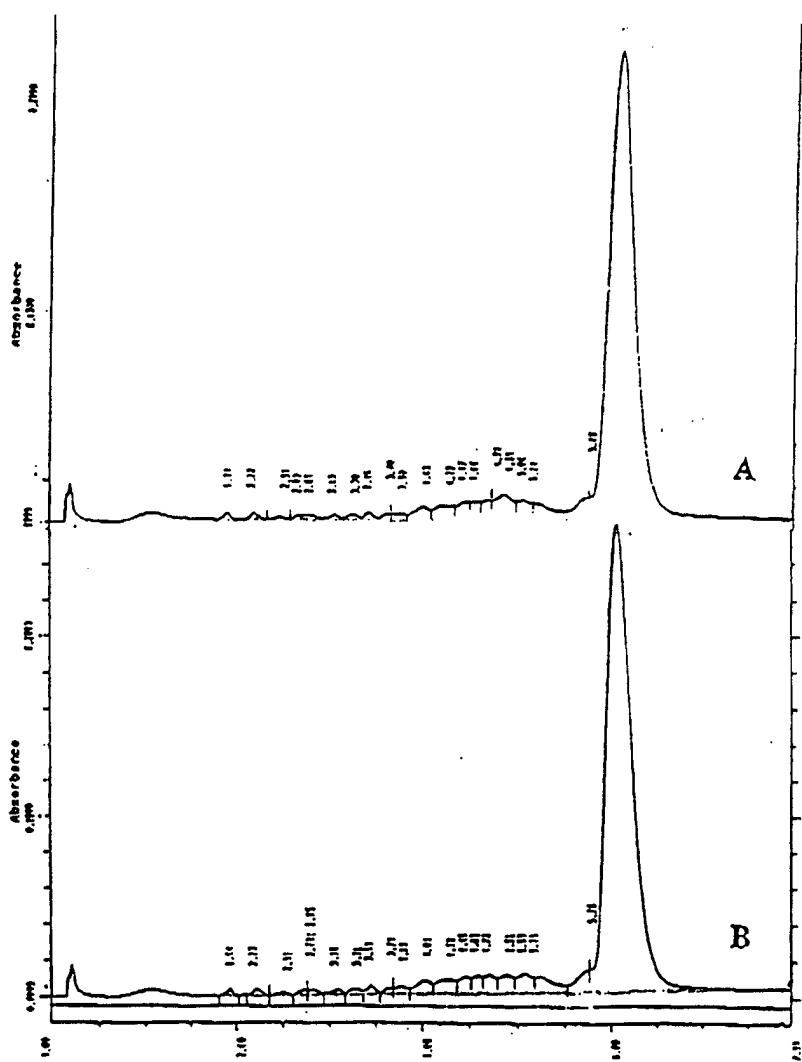
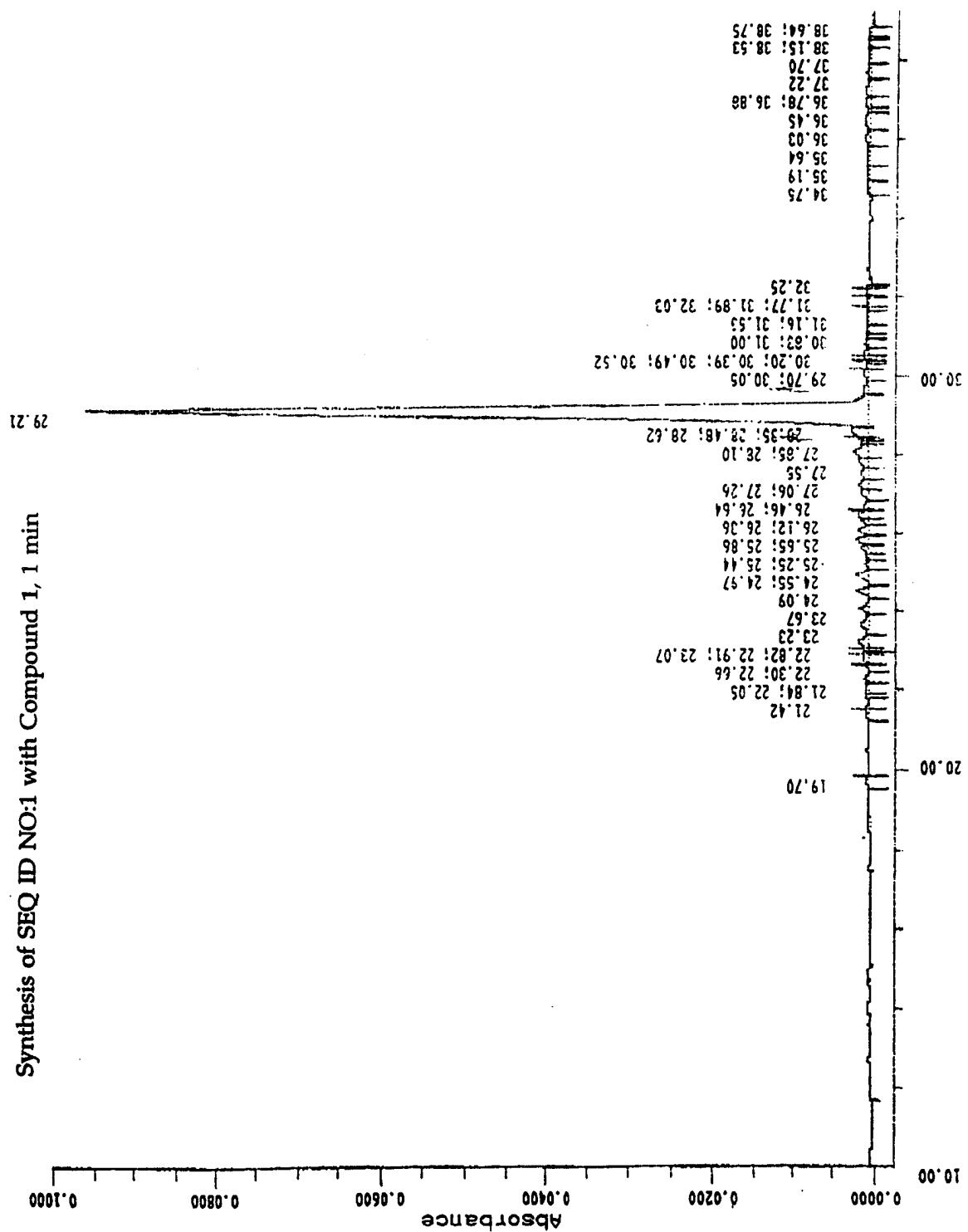


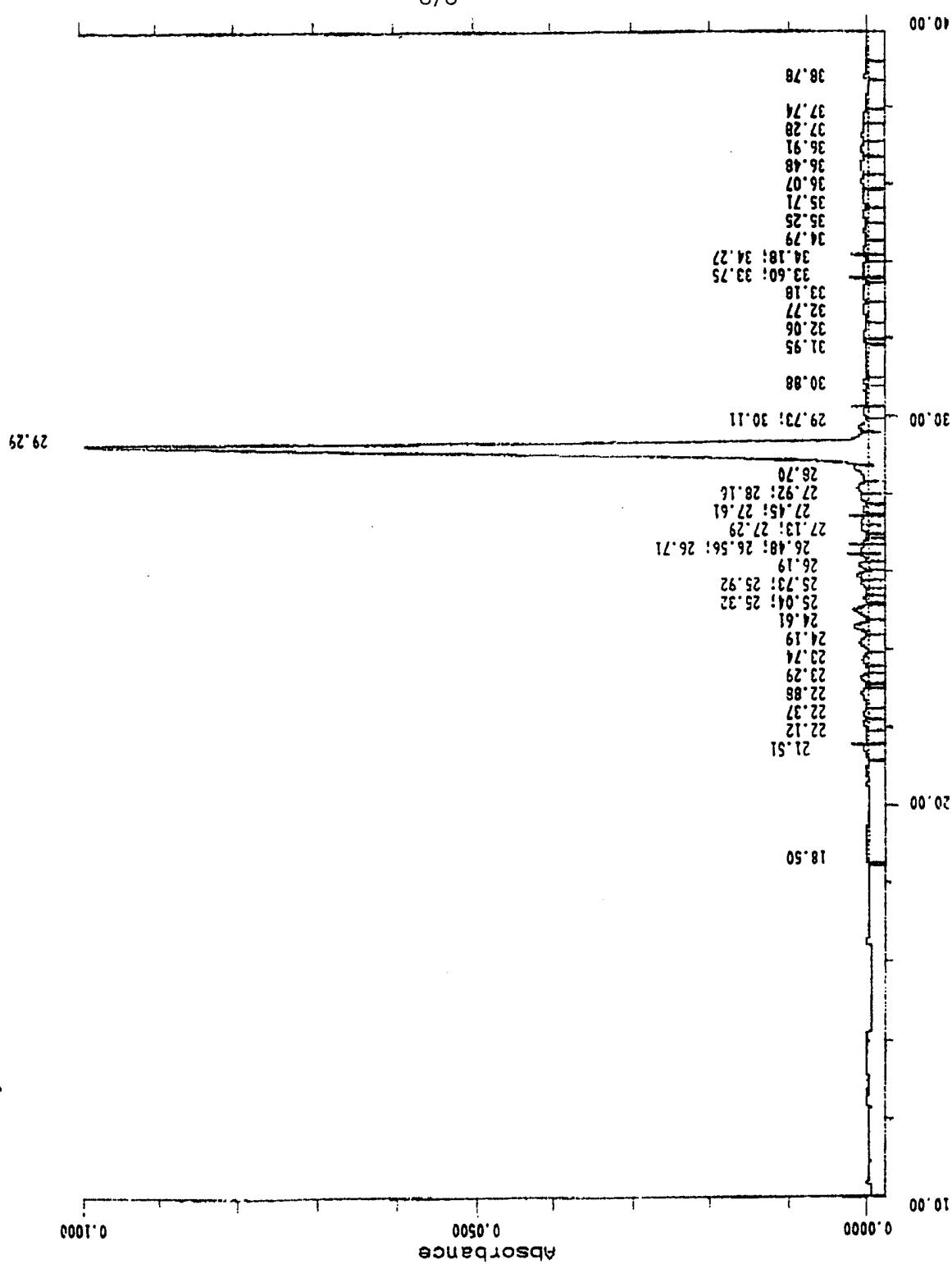
Figure 6. Ion-exchange HPLC of SEQ ID NO:1 prepared using 3-amino-1,2,4-diazole-5 thione (**1**) as sulfurizing reagent. HPLC conditions see General in Experimental Section.

Sulfurization was carried out at a concentration of 0.02 M (12 equiv) in pyridine-CH₃CN (1:9): A: 1 min (purity, 80.7%; total P=O, 2.48%; 0.103% P=O per step); B: 2 min (purity, 81.6%; total P=O, 2.38%; 0.099% P=O per step).

Figure 7
Synthesis of SEQ ID NO:1 with Compound 1, 1 min



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INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 98/10653

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 C07D277/00

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 C07D

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.
X	US 3 753 908 A (DE VRIES L ET AL) 21 August 1973 see claim 1 ---	1,2
X	US 5 502 066 A (HEINEMANN ULRICH ET AL) 26 March 1996 see claim W ---	3 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search 12 August 1998	Date of mailing of the international search report 25/08/1998
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 Scott, J

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10653

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CHEMICAL ABSTRACTS, vol. 115, no. 9, 2 September 1991 Columbus, Ohio, US; abstract no. 92173c, R.MAYER ET AL.: "Synthesis and Dimroth Rearrangement of Disubstituted 5-Imino-1,2,4-dithiazolidine-3-thiones." page 755; column 2; XP002074315 see abstract & J. PRAKT. CHEM., vol. 333, no. 1, 1991, pages 107-117, ---</p>	4,5
X	<p>CHEMICAL ABSTRACTS, vol. 118, no. 19, 10 May 1993 Columbus, Ohio, US; abstract no. 191647d, F.TITTELBACH ET AL.: "Cycloaddition-Elimination Reactions of Phenyl Isocyanate with Oxo and Thiono Derivatives of Aliphatic Substituted 5-Imino-1,2,4-Dithiazolidines and 3-Thiono-1,2,4-Thiadiazolidines." page 947; column 1; XP002074316 see abstract & J. PRAKT. CHEM., vol. 334, no. 8, 1992, pages 685-690, ---</p>	4,5
A	<p>XU Q ET AL: "USE OF 1,2,4-DITHIAZOLIDINE-3,5-DIONE (DTSNH) AND 3-ETHOXY-1,2,4-DITHIAZOLINE-5-ONE (EDITH) FOR SYNTHESIS OF PHOSPHOROTHIOATE-CONTAINING OLIGODEOXYRIBONUCLEOTIDES" NUCLEIC ACIDS RESEARCH, vol. 24, no. 9, 1 May 1996, pages 1602-1607, XP002037973 cited in the application see the whole document ---</p>	1-25
A	<p>XU Q ET AL: "EFFICIENT INTRODUCTION OF PHOSPHOROTHIOATES INTO RNA OLIGONUCLEOTIDES BY 3-ETHOXY-1,2,4-DITHIAZOLINE-5-ONE (EDITH)" NUCLEIC ACIDS RESEARCH, vol. 24, no. 18, 15 September 1996, page 3643/3644 XP002037974 see the whole document ---</p>	1-25
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10653

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	IYER R P ET AL: "THE AUTOMATED SYNTHESIS OF SULFUR-CONTAINING OLIGODEOXYRIBONUCLEOTIDES USING 3H-1,2-BENZODITHIOL-3-ONE, 1,1-DIOXIDE AS A SULFUR-TRANSFER REAGENT" JOURNAL OF ORGANIC CHEMISTRY, vol. 55, no. 15, 20 July 1990, pages 4693-4699, XP002046498 see the whole document -----	6-25

INTERNATIONAL SEARCH REPORT

Information on patent family members

In. national Application No
PCT/US 98/10653

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US 3753908	A 21-08-1973	NONE		
US 5502066	A 26-03-1996	DE 4320157 A	22-12-1994	
		EP 0629401 A	21-12-1994	
		JP 7025864 A	27-01-1995	