Synthesis of nucleoside and oligonucleoside dithiophosphates†‡

Xianbin Yang*[a] and Ellen Mierzejewski[b]

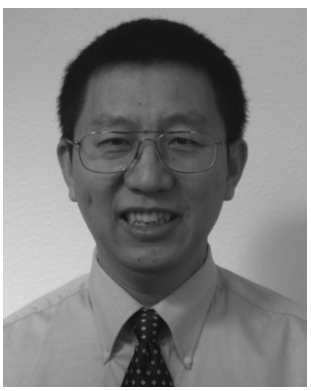
Received (in Montpellier, France) 30th October 2009, Accepted 13th January 2010
First published as an Advance Article on the web 19th February 2010
DOI: 10.1039/b9nj00618d

Nucleoside and oligonucleoside dithiophosphates are normal nucleotide and oligonucleotide mimics in which two nonbridging oxygen atoms at a phosphomonoester of a nucleotide or internucleotide phosphate moiety are replaced by two sulfurs. The nuclease resistance properties of nucleoside and oligonucleoside dithiophosphates have made them particularly interesting for the nucleic acid based therapeutics field. The high affinity for certain proteins has drawn some attention from aptamer-based diagnostics and therapeutic applications. The progress of nucleoside and oligonucleoside dithiophosphate development is summarized in this Perspective.

1. Introduction

Nucleotides and oligonucleotides (ODNs) are utilized in a wide range of applications in fields such as biotechnology, molecular biology, and medical diagnosis and therapy. Natural nucleotides and ODNs have been extensively used for functional genomics, selection, and determination of DNA sequences and site-directed mutagenesis. For example, Zamecnik and Stephenson[1,2] have demonstrated that ODNs can interact with specific stretches of RNA by the antisense approach. Recently, ODNs (called "aptamers") derived from large random libraries have also shown the ability to bind molecules with high affinity and specificity.[3,4] Additionally, the spectrum of nucleotide and ODN applications can be broadened by chemically modifying their structures. Over the past two decades, besides nucleoside and oligonucleoside monothiophosphates which have proven to be valuable analogs for studying many biological and biochemical processes,[5,6] among the most intriguing analogs of nucleotides and ODNs are the dithiophosphate congeners in which two nonbridging oxygen atoms at a phosphomonoester of a nucleotide or internucleotide phosphate moiety are replaced by two sulfurs.[7–9] Like the phosphate (Fig. 1a), the dithiophosphate (Fig. 1b)

[a] *AM Biotechnologies, 12521 Gulf Freeway, Houston, Texas, USA. E-mail: xianbin.yang@thioaptamer.com, xianbin@hotmail.com; Fax: 832 379 0294; Tel: 832 379 2175*
[b] *AM Biotechnologies, 12521 Gulf Freeway, Houston, Texas, USA. E-mail: ellen@thioaptamer.com; Fax: 832 379 0294; Tel: 832 379 2176*
† Dedicated to the 70th birthday of Professor Wojciech Stec.
‡ This article is part of a themed issue on Biophosphates.



Xianbin Yang

Xianbin Yang is Director of Research and Development at AM Biotechnologies in Houston, where he has worked since 2007. He is also an adjunct faculty member at the University of Houston Clear Lake. He studied chemistry at the School of Pharmaceutical Sciences at Beijing Medical University, where he obtained his B.S. and M.S. degrees with Professor Lihe Zhang. His doctorate was supervised by Professor Wojciech Stec from the Center of Molecular and Macromolecular Studies of the Polish Academy of Sciences. In 1998, he joined Dr. David Gorenstein's laboratory as a postdoctoral fellow at the University of Texas Medical Branch (UTMB) in Galveston. He became a faculty member at the Department of Biochemistry and Molecular Biology at UTMB in 2004. His interests primarily center around nucleic acid chemistry, especially the chemistry and biological applications of oligonucleoside phosphorothioates and oligonucleoside phosphorodithioates. He is also very interested in the application of NMR techniques for structure evaluation.

Ellen Mierzejewski

Ellen Mierzejewski is a research chemist at AM Biotechnologies where she has worked since 2009. She graduated from the University of Virginia with a B.S. in Chemistry and continued her studies at Yale University where she received a Masters degree in Chemistry. Before working for AM Biotechnologies, her research was physical chemistry based working with Dr. Charles Schmuttenmaer during her graduate studies, and Dr. Brooks Pate during her undergraduate studies. Over the past year she has worked alongside Dr. Yang to advance methods relating to the synthesis and purification of modified DNA and RNA oligonucleotides.

is isostructural and isopolar. Although *O*,*O*-dialkyl esters of dithiophosphate acid and their salts have been used for several decades as rubber and engine oil antioxidants and as important substrates in the pesticide industry,[10] recent developments in nucleoside and oligonucleoside dithiophosphate chemistry, biochemical, and biological studies have shown very interesting and potentially useful results. These include observations that adenosine 3′,5′-cyclic dithiophosphate (cAMPS2)[11] has cAMP antagonist properties and is a competitive inhibitor of cAMP-dependent protein kinase,[12] and that oligonucleoside dithiophosphate (PS2-ODN) activates RNase H *in vitro*,[13] strongly inhibits human immunodeficiency virus (HIV) reverse transcriptase,[14] induces B-cell proliferation and differentiation,[15] is completely resistant to hydrolysis by various nucleases,[14] and binds to protein targets in the form of dithiophosphate aptamers (thioaptamers).[16] This Perspective will focus on the chemistry and some of the properties of deoxyribonucleoside and oligodeoxyribonucleoside dithiophosphates with the type of structure shown in **1b** of Fig. 1. We will not cover several other kinds of dithiophosphate analogs such as nucleoside 5′-*O*-(1,3-dithiotriphosphates),[17] nucleoside 5′-*O*-(1,2-dithiotriphosphates),[18] nucleoside phosphofluorodithioates,[19] *O*-dithiophosphonopeptides,[20] and phosphorodithioate phospholipid derivatives.[21] The structural studies of the PS2-ODNs will also not be discussed here.[22–28]

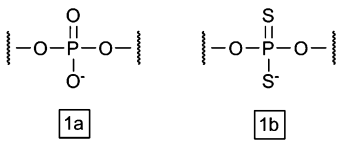


Fig. 1 Phosphate (**1a**) and dithiophosphate (**1b**).

2. Nucleoside dithiophosphates

2.1 Nucleoside 3′- and 5′-dithiophosphates

Nucleoside 3′-dithiophosphates **2a–d** and 5′-dithiophosphates **3a–d** (Fig. 2), bearing two sulfur atoms at the nonbridging positions of the phosphomonoester moiety,[29–34] have been prepared for biochemical and biological evaluations and their syntheses highlighted some fundamental problems connected with their preparation. The dithiophosphates

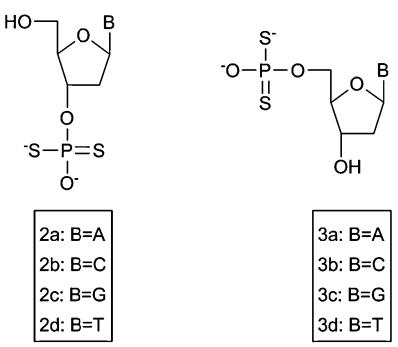
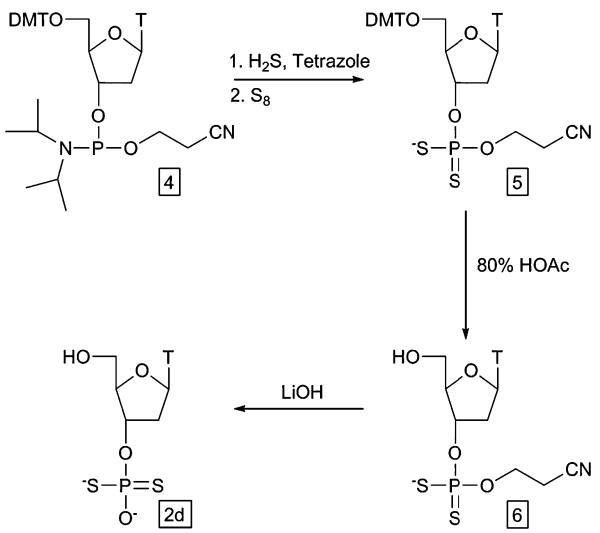


Fig. 2 Structures of nucleoside 3′-dithiophosphates (**2a–d**) and 5-dithiophosphates (**3a–d**).

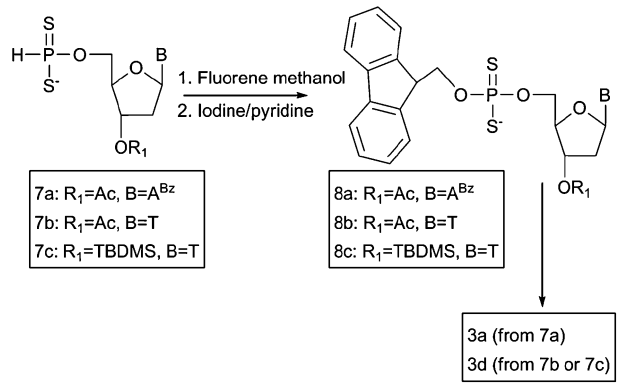
2a–d and **3a–d** are stable in dry form for a couple of months at −30 °C while at low pH in solution form, they are quite unstable.

2.1.1 Synthesis *via* a phosphoramidite approach. The phosphoramidite approach has been widely used in nucleic acid chemistry in the synthesis of ODNs.[35–37] This approach was first studied more than a decade ago to synthesize the thymidine 3′-dithiophosphate **2d** starting from a commercially available phosphoramidite **4**, which is described in Scheme 1.[30] Phosphorodithioate **5** was prepared in a one-pot reaction by treatment of thymidine phosphoramidite **4** with H_2S and tetrazole followed by elemental sulfur. Removal of the 5′-dimethoxytrityl (DMT) group with 80% acetic acid (HOAc) yields compound **6**. Experience has shown it difficult to cleave the β-cyanoethyl protecting group of the dithiophosphate diester **6** for conversion into the desired dithiophosphate monoester **2d** with sodium hydroxide or concentrated ammonium hydroxide at room temperature or 55 °C. Although an acceptable result was obtained when **6** was treated with aqueous 0.4 M LiOH at reflux for 30 min to generate the desired dithiophosphate **2d**, the strong alkaline conditions required for removal of the β-cyanoethyl group would not be compatible with the synthesis of other nucleosides. It is worth emphasizing that the triethylammonium salt form of compound **2d** decomposes slowly to thymidine in methanol and water. Based upon TLC analysis, compound **2d** does not decompose when dissolved in a phosphate buffered aqueous solution. Experimentally, the ^{1}H-decoupled ^{31}P NMR spectrum with a single peak at δ 88–90 ppm indicates a dithiophosphate monoester characteristic structure such as compound **2d**, while the ^{1}H-decoupled ^{31}P NMR spectrum with a single peak at δ 110–116 ppm indicates a dithiophosphate diester characteristic structure such as compounds **5** and **6**.

2.1.2 Synthesis *via* an *H*-phosphonodithioate approach. This approach was invented by Caruthers' laboratory to prepare the adenosine and thymidine 5′- and 3′-dithiophosphates.[30]



Scheme 1 Synthesis of thymidine 3′-dithiophosphate *via* a phosphoramidite approach.



Scheme 2 Synthesis of nucleoside 5′-dithiophosphate *via* an *H*-phosphonodithioate approach.

The synthesis of the nucleoside 5′-dithiophosphates is depicted in Scheme 2 and was accomplished through the coupling of nucleoside *H*-phosphonodithioate **7** with 9-fluorenemethanol under oxidation conditions to form nucleoside 9-fluorenemethyl phosphonodithioate **8**, which was then converted to nucleoside 5′-dithiophosphates **3** *via* concentrated ammonium hydroxide followed by HPLC purification in *ca.* 30% yield based on the *H*-phosphonodithioate **7**. This approach involved nucleoside *H*-phosphonodithioate **7** as an intermediate which resulted from the hydrogen sulfenolysis of the nucleoside bistriazolophosphoramidite generated in a one-pot reaction by the treatment of the properly protected nucleoside with *in situ* formed tristriazolophosphine. It is worth mentioning that the 9-fluorenemethylphosphonodithioate diester **8** with a 3′-*O*-*tert*-butyldimethylsilyl (TBDMS) protecting group proved to be labile to *tert*-butylammonium fluoride (TBAF) meaning that silyl protecting groups could not be used in this synthesis scheme. This approach was also used to synthesize **2a** and **2d**. The characteristic ^{31}P NMR chemical shift for the *H*-phosphonodithioate type of compound **7** is at δ 84–87 ppm.

2.1.3 Synthesis *via* an *H*-phosphonothioate approach. The synthesis of nucleoside dithiophosphate *via* *H*-phosphonothioate chemistry has been explored in the past decade by several investigators.[31–34] This approach serves as a convenient alternative for the synthesis of nucleoside dithiophosphates. To the best of our knowledge, the conversion of dialkyl *H*-phosphonothioate diester into dialkyl *H*-phosphonodithioate diester *via* elemental sulfur was first reported by Wada in 1990.[33] However, Wada did not report about the formation of the dithiophosphate monoester form.[33] A new *H*-phosphonothionylating reagent, 9-fluorenemethyl *H*-phosphonothioate **9** (Fig. 3), which can be used to efficiently prepare nucleoside *H*-phosphonothioate diester **10** and to introduce the 5′-dithiophosphate function to ODNs, was advocated by Seeberger in Caruthers' laboratory[38] and independently developed by Jankowska in 1997[32]

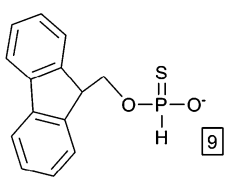
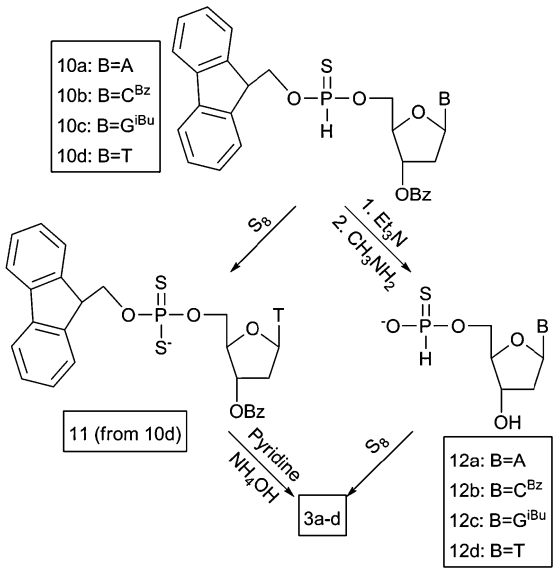


Fig. 3 The structure of 9-fluorenemethyl *H*-phosphonothioate (**9**).

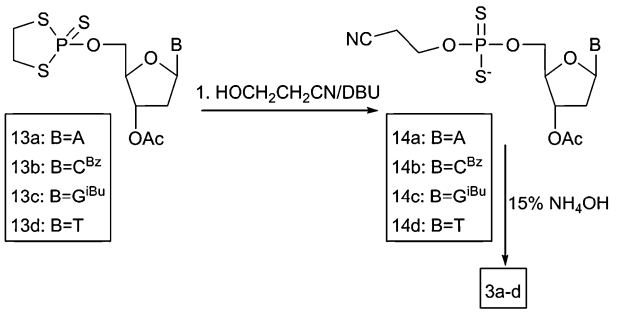


Scheme 3 Synthesis of nucleoside 5′-dithiophosphate *via* an *H*-phosphonothioate approach.

(Scheme 3). This reagent has been reported to successfully synthesize nucleoside 5′-dithiophosphates **3a–d**. Two approaches have been studied in Kraszewski's laboratory to prepare compounds **3a–d**. The first approach, treatment of the *H*-phosphonothioate **10d** with elemental sulfur in the dichloromethane containing lutidine, furnished a clear formation of the dithiophosphate **11** in 91% isolated yield. Removal of the protecting groups from **11** with pyridine/concentrated ammonia (v/v, 1 : 1) afforded the desired thymidine dithiophosphate **3d** as a predominant product (80%, ^{31}P NMR), but the reaction mixture also contained a variety of by-products such as dithiophosphate salt and thymidine phosphorothioate. Alternately, nucleoside 5′-*H*-phosphonothioate **10** can be reacted first with triethylamine to remove the 9-fluorenemethyl group, then treated with 50% aqueous methylamine to remove the remaining protecting group, such as the N^2-isobutylryl group of G of **10c**. Removal of this group forms the unprotected nucleoside 5′-*H*-phosphonothioate derivative **12**, which was converted under mild sulfurization conditions to the corresponding nucleoside 5′-dithiophosphate **3**.

The main advantages of this alternative strategy from compound **10** *via* **12** to compound **3** are the following: (1) the degradation of the dithiophosphate function in the nucleotide **3** is minimized due to its generation in the last synthetic step; (2) the sulfurization is rapid and occurs under mild reaction conditions; (3) the starting material, nucleoside *H*-phosphonothioate **10**, is stable, easy to handle, and readily accessible from standard or commercially available nucleosidic precursors.

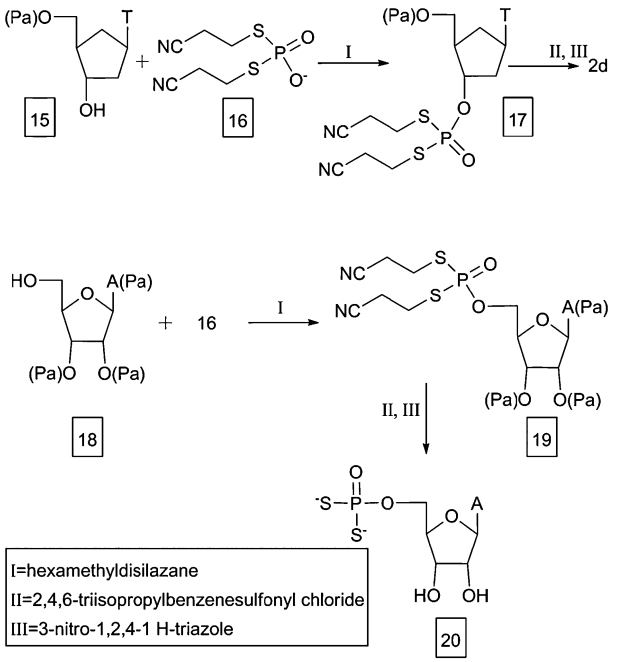
2.1.4 Synthesis *via* a dithiaphospholane approach. The dithiaphospholane method was an extension of the oxathiaphospholane method, which was first introduced for the stereocontrolled synthesis of oligonucleoside phosphorothioate.[39,40] Protected nucleoside 5′-*O*-(2-thio-1,3,2-dithiaphospholanes) **13a–d** (Scheme 4) in acetonitrile were reacted at room temperature with β-cyanoethanol (3-hydroxypropionitrile) in the presence of an equimolar amount of 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) to form the *O*,*O*-dialkyl phosphorodithioates **14a–d**. This was



Scheme 4 Synthesis of nucleoside 5′-dithiaphosphate *via* a dithiaphospholane approach.

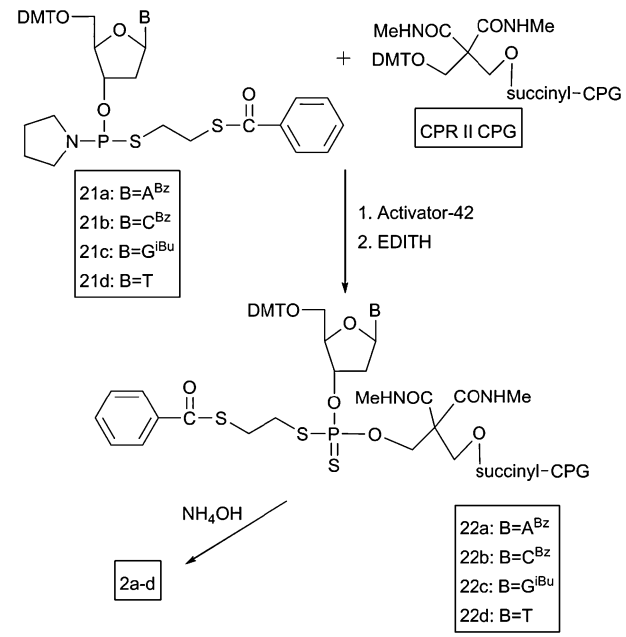
confirmed by the chemical shift at δ 117 ppm from ^{31}P NMR analysis. The crude reaction mixtures were then treated overnight at 55 °C with an excess of 15% aqueous ammonia to form the crude **3a–d** as single products based on ^{31}P NMR analysis. The crude **3a–d** were purified by means of ion-exchange chromatography on Sephadex A-25 and isolated in roughly 50% yield. Another set of nucleoside 3′-dithiophosphates **2a–d** were similarly prepared by the same synthetic approach starting from 3′-*O*-(2-thio-1,3,2-dithiaphospholane) derivatives of 5′-*O*-DMT-nucleosides with acyl-protected reactive amino groups at nucleobases. The yields of isolated nucleoside dithiophosphates **2a–d** are in the similar range of 50–53%. The final isolation on a Sephadex column may account for the low isolated yield in the final step. Interestingly, it was also reported that the 1,3,2-dithiaphospholane derivative reacts with TBAF to form phosphonofluorodithioate[19]

2.1.5 Synthesis *via* a phosphotriester approach. The phosphotriester method,[41,42] which was first developed by Michelson and Todd,[43] is still regarded as the method of choice for large-scale synthesis of ODNs. This approach was very recently introduced by Reese to synthesize nucleoside dithiophosphates.[44] Briefly, treatment of 5′-*O*-phenoxyacetyl-thymidine **15** with the phosphorodithioate salt **16**, which was obtained by allowing ammonium phosphinate to react with *N*-[(β-cyanoethyl)sulfanyl]-phthalimide in the presence of hexamethyldisilazane, gave the intermediate phosphotriester **17** in high yield. The phosphotriester formation was catalyzed by 2,4,6-triisopropylbenzenesulfonyl chloride and 3-nitro-1,2,4-1 *H*-triazole in the pyridine solution. The product **17** was treated first with an excess each of chlorotrimethylsilane and 1,1,3,3-tetramethylguanidine (TMG) in acetone solution at 60 °C to remove both β-cyanoethyl protecting groups and then with aqueous ammonia to remove the phenoxyacetyl protecting groups. Thymidine dithiophosphate **2d** was obtained as its 1,1,3,3-tetramethylguanidinium ($TMGH^+$) salt in quantitative yield. In the same way, 6-*N*,2′-*O*,3′-*O*-tri(phenoxyacetyl)adenosine **18** was converted into the intermediate triester **19** in almost quantitative yield. Following the deblocking procedure, ribonucleoside (adenosine) 5′-*O*-dithiophosphate **20** was obtained as its $TMGH^+$ salt, also in high yield (Scheme 5).



Scheme 5 Synthesis of nucleoside dithiophosphate *via* a phosphotriester approach.

2.1.6 Synthesis *via* a thiophosphoramidite approach. The aforementioned synthesis of nucleoside dithiophosphate is carried out in solution. Very recently, the availability of thiophosphoramidite building blocks[45] from AM Biotechnologies (www.thioaptamer.com) along with the CPR II CPG solid support from Glen Research (www.glenresearch.com) has made the synthesis of nucleoside and ODN 3′-dithiophosphates simple and easy. Following 3% trichloroacetic acid (TCA) detritylation of a CPR II linked to the solid support CPG (Scheme 6), the thiophosphoramidite **21** is coupled directly to the free hydroxyl group on the support using an activator such as 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole (Activator-42, commercially available from Sigma-Aldrich). The resulting thiophosphite triester is then sulfurized by either 3-ethoxy-1,2,4-dithiazolidine-5-one (EDITH) or 3-[(*N*,*N*-dimethyl-aminomethylidene)amino]-3*H*-1,2,4-dithiazole-5-thione (DDTT) reagent. Deprotection under standard



Scheme 6 Synthesis of 3′-dithiophosphate *via* a 3′-thiophosphoramidite approach.

conditions with concentrated NH_4OH will eliminate the Solid CPR II to yield a 3′-dithiophosphate **2**. This approach, *via* the thiophosphoramidite building blocks, provides a new and efficient way to synthesize nucleoside 3′-dithiophosphates. Very similarly, this approach can be used to synthesize ODNs with 3′-*O*-dithiophosphate (www.glenresearch.com).

2.2 Chemical and biological studies

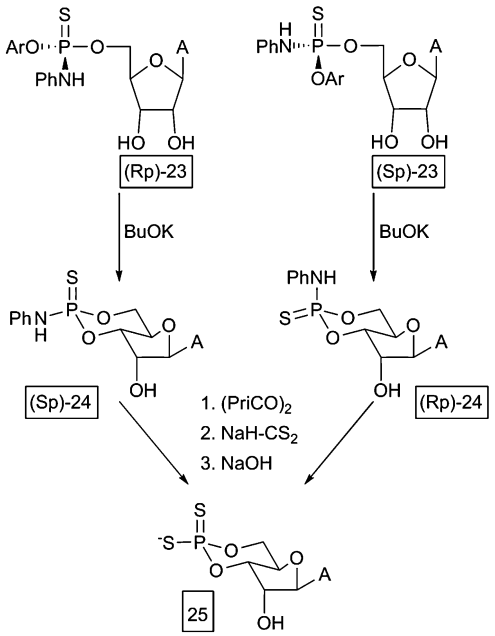
To provide important insights regarding nucleoside dithiophosphates as therapeutic agents and on their behavior in potential pharmaceutical applications, the hydrolysis of **2d** under acidic conditions was studied. When monitored after 24 h, the hydrolysis occurred most rapidly at low pH such as pH 3.5.[30,44] Hydrolysis slowed significantly with an increase in pH.

Nucleoside dithiophosphates **2d**, **3a**, and **3d** showed modest inhibition of avian myeloblastosis virus reverse transcriptase and failed to inhibit human immunodeficiency virus reverse transcriptase, alkaline phosphatase, T4 polynucleotide kinase, and DNA polymerase I Klenow fragment.[30] None of the nucleoside dithiophosphates **2b**, **3a**, **3b**, and **3d** were active against HIV-1 or HIV-2 at subtoxic concentration.[29] Interaction studies of thymidylate synthase with the 2′-deoxyuridine dithiophosphate (dUMPS2) and **3d** indicated that dUMPS2 was a good substrate, while **3d** was a classic competitive inhibitor.[46]

2.3 Nucleoside 2′,3′- and 3′,5′-cyclic dithiophosphates

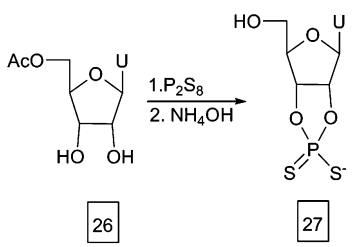
Nucleoside cyclic phosphates and their analogs are important intermediates in various biochemical processes. For example, nucleoside 3′,5′-cAMP and its derivatives act as mediators[47] of hormone action and modulators of enzymatic activity,[48] and an (S_p)-octyl-8-chloroadenosine 3′,5′-cAMP analog strongly induced growth inhibition and differentiation in human leukemia HL-60 cells.[49] 2′,3′-cyclic nucleotides are intermediates and substrates of ribonuclease (RNase)-catalyzed reactions.[50] To better understand these processes, various modified nucleoside cyclic phosphate analogs have been prepared to probe the stereochemical and mechanistic aspects of enzymatic reactions and their inhibition.[11,51–59] Compared to other modified nucleoside cyclic phosphates, nucleoside cyclic dithiophosphates are a largely unexplored class of nucleotide analogs.

2.3.1 Synthesis of nucleoside 3′,5′-cyclic dithiophosphates. Adenosine 3′,5′-cyclic dithiophosphate (cAMPS2, 25) was first synthesized by Baraniak and Stec *via* adenosine cyclic 3′,5′-phosphoranilidothioate (S_p)-24 or (R_p)-24 as depicted in Scheme 7.[11] Briefly, each individual diastereoisomer of compound **23** in the reaction with BuOK in dimethylformamide (DMF) solution was stereospecifically converted into the diastereomerically pure P-chiral cyclic nucleoside thiophosphate analogs **24** *via* the Stec reaction.[11,60] Following 2′-hydroxy function protection *via* isobutyric anhydride, the resulting $O^{2\prime}$-isobutyryladenosine cyclic 3′,5′-thiophosphates upon treatment with NaH-CS_2, gave $O^{2\prime}$-isobutyryladenosine cyclic 3′,5′-dithiophosphate, which under work-up procedure was protected to afford the desired dithiophosphate **25**. The final compound was confirmed by ^{31}P NMR with the chemical shift at δ 110 ppm. Importantly, cAMPS2 has antagonist properties and is a competitive inhibitor of cAMP-dependent protein kinase.[12] More recently, Reese *et al.* reported a simple method to synthesize cAMPS2 in better yield.[61]

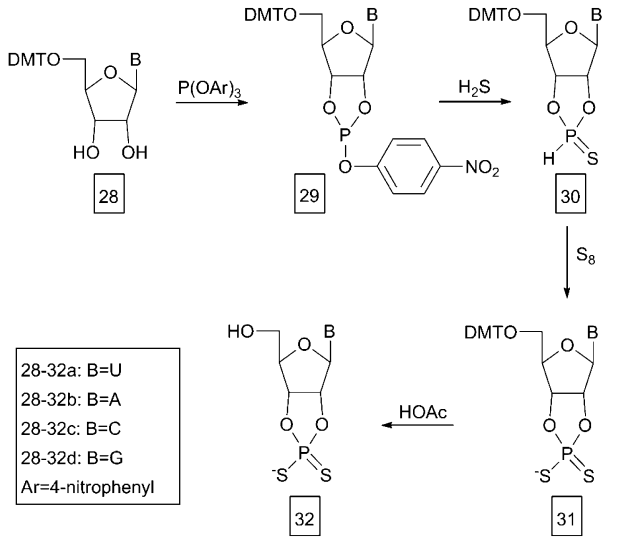


Scheme 7 Synthesis of adenosine 3′,5′-cyclic dithiophosphate.

2.3.2 Synthesis of nucleoside 2′,3′-cyclic dithiophosphates. The first example of the nucleoside 2′,3′-cyclic dithiophosphate family is the uridine 2′,3′-cyclic dithiophosphate **27** which was obtained by reaction of 5′-*O*-acetyluridine **26** and P_2S_8 at elevated temperature followed by treatment with ammonium (Scheme 8). **27** has been demonstrated as a substrate for pancreatic ribonuclease.[62] Synthesis of all the four base analogs of 2′,3′-cyclic dithiophosphate was accomplished *via* the *H*-phosphonothioate approach.[63] As described in Scheme 9, reaction of tri(4-nitrophenyl) phosphine with 5′-protected nucleoside **28** gives the phosphite intermediate **29** with high yield. The intermediate **29** was treated with H_2S to produce the *H*-phosphonothioate intermediate followed by reaction with elemental sulfur to produce the 2′,3′-phosphorodithioate **31**. After removal of the 5′-protecting group with 80% acetic acid, the final compound, nucleoside 2′,3′-cyclic dithiophosphate **32**, was isolated by simple silica gel chromatography in 95% yield. All transformations involved can be carried out as a one-pot reaction and are compatible with nucleoside substrates bearing unprotected amino functions. The method is very efficient and experimentally simple.



Scheme 8 Synthesis of uridine 2′-3′-cyclic dithiophosphate.

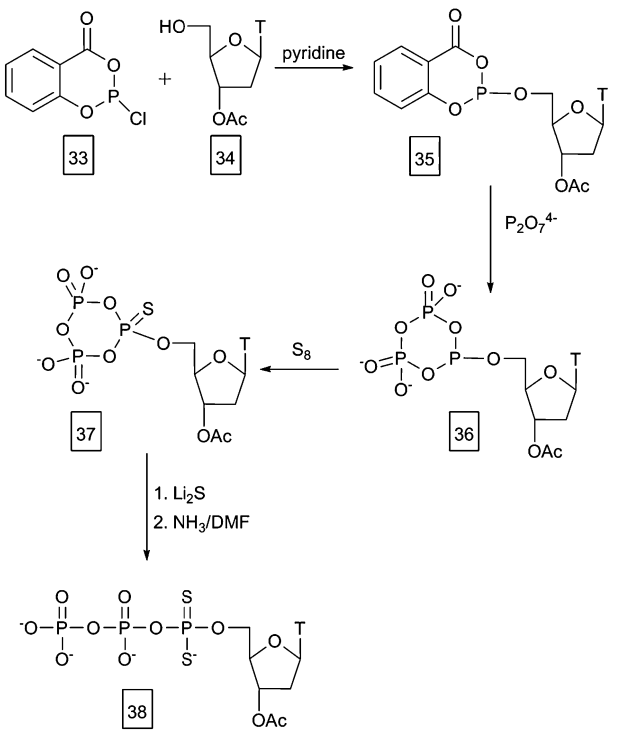


Scheme 9 Synthesis of nucleoside 2′,3′-*O*,*O*-cyclic phosphorodithioates *via* *H*-phosphonothioate.

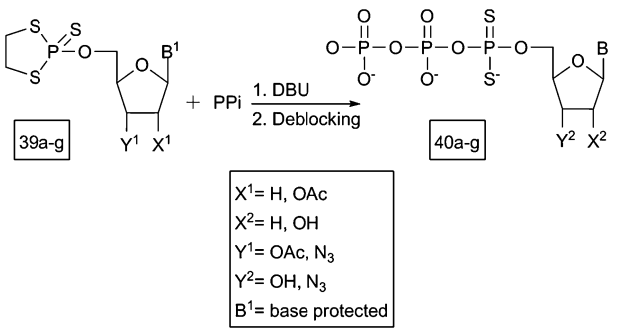
2.4 Nucleoside 5′-*O*-(1,1-dithiotriphosphate)s

2.4.1 Synthesis *via* a phosphite approach. The thymidine 5′-*O*-(1,1-dithiotriphosphate) was synthesized by Ludwig and Eckstein.[17] 3′-*O*-acetyl-thymidine **34** was reacted with 2-chloro 4*H*-1,3,2-benzodioxaphosphorin-4-one (salicyl chlorophosphite, **33**) in DMF containing pyridine, which was subsequently reacted with pyrophosphate in a double displacement process to form a cyclic intermediate, P^2,P^3-dioxo-P^1-5′-nucleosidylcyclotriphosphite **36**. Oxidation of the cyclic intermediate with elemental sulfur followed by Li_2S selectively gave nucleoside 5′-*O*-(1,1-dithiotriphosphate) **38** *via* α-thiotriphosphate **37**. However, when performed in pyridine/dioxane, this reaction led to a mixture of the nucleoside 5′-*O*-(1,3-dithiotriphosphate) and the nucleoside 5′-*O*-(1,1-dithiotriphosphate). Because a phosphitylating reagent does not discriminate between primary and secondary alcohol, protection of the 2′- and/or 3′-hydroxyl is necessary. Although the yields were low (13–22%), compound **38** and guanosine 5′-*O*-(1,1-dithiotriphosphate) were the first published examples of nucleoside 5′-*O*-(1,1-dithiophosphate) analogs. The thymidine 5′-*O*-(1,1-dithiotriphosphate) **38** was found not to be a substrate for the Klenow DNA polymerase (Scheme 10).

2.4.2 Synthesis *via* a dithiaphospholane approach. Recently, a new synthesis *via* a dithiaphospholane approach was developed for the preparation of nucleoside 5′-*O*-(1,1-dithiotriphosphates) **40a–g**. As shown in Scheme 11, appropriately protected nucleoside 5′-*O*-(2-thio-1,3,2-dithiaphospholanes) **39a–g** reacted with inorganic pyrophosphate in the presence of a strong base catalyst (DBU) to give nucleoside 5′-*O*-(1,1-dithiotriphosphates) derivative **40a–g**. After completion of the reaction, the crude mixtures were treated with 25% aqueous ammonia to remove protecting groups. The final products **40a–g** were isolated and purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column with aqueous triethylammonium bicarbonate (TEAB) as eluent. The final yields are in the range of 12–26%. Compounds **40a–g** showed modest antivirial activity against HIV-1 and HIV-2 replication in CEM cells. The AZT and deoxyadenosine derivatives were found to be inhibitors of HIV reverse transcriptase.



Scheme 10 Synthesis of 5′-*O*-(1,1-dithiotriphosphates) *via* a phosphite approach.



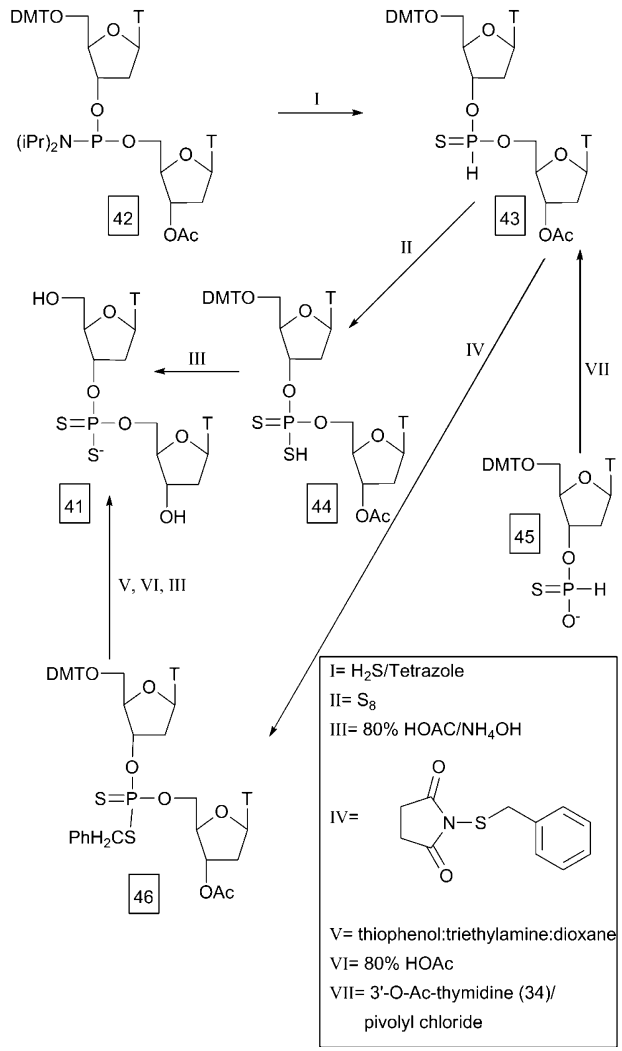
Scheme 11 Synthesis of 5′-*O*-(1,1-dithiotriphosphates) *via* a dithiaphospholane approach.

2.5 Dinucleoside and oligonucleoside dithiophosphates

The past two decades have seen an upsurge of interest in nucleic acid-based drug development. This is mainly due to the realization that modified ODNs, with improved properties, can be used for sequence specific control of gene expression *via* antisense and siRNA mechanisms and to modulate protein functions *via* an aptamer mechanism. Replacing two nonbridging oxygen atoms in the phosphate moiety by two sulfur atoms results in a class of backbone-modified PS2-ODNs that are stable toward nucleases, display enhanced hydrophobicity and have promising biological properties as mentioned early. In particular, we and others have found that PS2-ODNs bind proteins with a higher affinity than their phosphodiester analogs,[64,65] suggesting that PS2-ODNs may have additional utility in the form of sulfur-modified phosphate ester aptamers (thioaptamers)[16,65] in therapeutic and diagnostic applications.[14,16] Nucleoside dithiophosphate dimers have been prepared in

several ways using *H*-phosphonothioate, *H*-phosphonodithioate, dithiaphospholane, phosphorodithioate triester, phosphorodiamidite, and thiophosphoramidite approaches. Most of the oligonucleosides containing PS2 linkage(s) and/or PS2-ODNs have been prepared *via* thiophosphoramidite chemistry and phosphorodithioate triester.

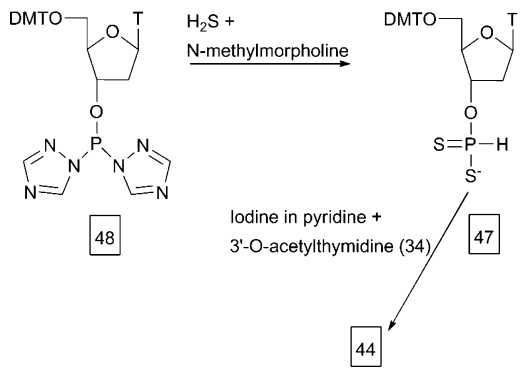
2.5.1 Synthesis of PS2 dimers or trimers *via* an *H*-phosphonothioate. The first synthesis of dithymidine dithiophosphate **41** was carried out in Caruthers' laboratory *via* an *H*-phosphonothioate method.[8] As described in Scheme 12, the key intermediate is the dithymidine *H*-phosphonothioate **43** which can be obtained by bubbling H_2S into an acetonitrile solution of dithymidine phosphoramidite **42** in the presence of tetrazole. Conversion of the *H*-phosphonothioate **43** to protected dithiophosphate **44** was achieved by elemental sulfur in dichloromethane. The final product **41** was obtained in good yield after the removal of protection groups by NH_4OH. Alternately, the *H*-phospohonothioate **43** can be obtained *via* the condensation of thymidine 3′-*H*-phosphonothioate **45** with 3′-*O*-acetylthymidine **34** in the presence of pivaloyl chloride. A similar procedure was also reported by Porritt.[66] However, the reaction conditions for the latter approach need to be properly chosen.[67] In line with the *H*-phosphonothioate approach, Dreef described an analogous method for the synthesis of *S*-benzyl protected dithiophosphate **46** in almost quantitative yield by using *S*-benzyl thiosuccinimide in the presence of *N*,*N*-diisopropylethylamine (DIEPA). The benzyl protection group was removed by treatment with a mixture of thiophenol:triethylamine:dioxane (1 : 2 : 2, v/v/v) at room temperature for 20 h.[68] Removal of the 5′-DMT by 80% acetic acid and 3′-acetyl by NH_4OH led to dithymidine dithiophosphate **41** in good yield. More recently, this approach was used on solid support to efficiently synthesize dithiophosphate octamer [$(TpS_2)_7T$] by using bis(2,6-dimethylphenyl) phosphorochloridate as a coupling agent.[69]



Scheme 12 Synthesis of dithymidine dithiophosphate *via* an *H*-phosphonothioate approach.

2.5.2 Synthesis of PS2 dimers *via* an *H*-phosphonodithioate. The *H*-phosphonodithioate approach for generating the dithiophosphate internucleotide linkage is *via* the oxidative coupling of the nucleoside 3′-*H*-phosphonodithioate with the nucleoside 5′-hydroxyl group. For example, the protected key intermediate thymidine 3′-*H*-phosphonodithioate **47** was prepared by passage of H_2S through a reaction mixture of the 3′-phosphorobistriazolide **48** (Scheme 13). The dithymidine dithiophosphate **44** was prepared in good yield by treatment of **47** and 3′-*O*-acetylthymidine **34** with iodine in pyridine.[70] This method is mild and versatile for synthesis of cholesterol-nucleoside and amino acid-nucleoside hybrids.[71] Independently, Porritt and Reese have converted the triethylammonium salt of 5′-*O*-(p-phenylxanthen-9-yl)thymidine 3′-phosphonodithioate into the dithymidine dithiophosphate analog.[66] In addition, Porritt also reported the synthesis of a corresponding trinucleoside dithiophosphate analog, using the 2,4-dinitrobenzyl group to protect the phosphorodithioate internucleotide linkages.[72] Using this approach, a dodecanucleotide containing a *cis–syn* thymine dimer **49** in Fig. 4 with a phosphorodithioate linkage was synthesized on a solid support.[73]

2.5.3 Synthesis of PS2-ODNs *via* a dithiaphospholane approach. In the course of studies on new methodology for the stereocontrolled synthesis of oligonucleoside phosphorothioate (PS-ODN) with a predetermined sense of chirality at each phosphorothioate function, Stec's laboratory has developed the oxathiaphospholane strategy.[40,74] As a natural extension of the chemistry, Okruszek *et al.* have adapted this methodology to the synthesis of PS2-ODN by replacing



Scheme 13 Synthesis of dithymidine dithiophosphates *via* an H-phosphonodithioate.

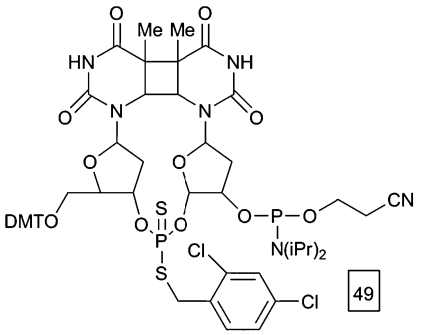
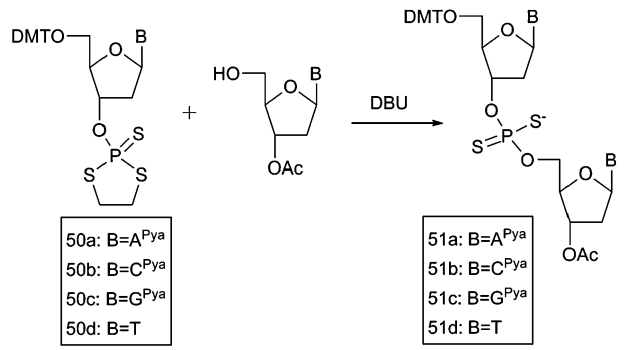


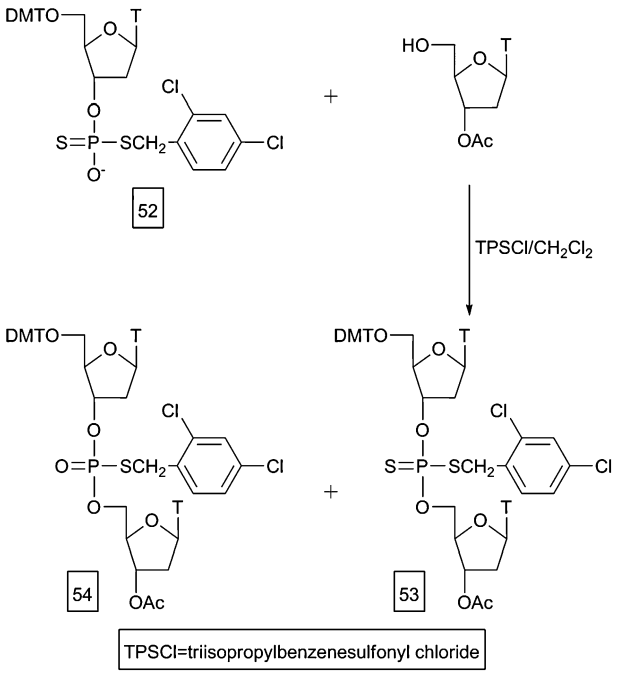
Fig. 4 The structure of phosphoramidite containing the *cis–syn* thymine dimer (**49**).

nucleoside 3′-*O*-(2-thiono-1,3,2-oxathiaphospholanes) with corresponding nucleoside 3′-*O*-(2-thiono-1,3,2-dithiaphospholanes **50a–d**.[75] Therefore, the dithiaphospholanes **50a–d** in Scheme 14 reacted with the 5′-OH of the protected nucleoside in the presence of DBU to form nucleoside dithiophosphate **51** as shown in Scheme 14. Importantly, this approach has been adapted to the requirements of an automated solid-phase ODN synthesis with a relatively short condensation step (5 min) and reasonable step coupling yield (>95%). *N*-methylpyrolidin-2-ylidenyl (Pya) was found to be the group of choice for the protection of reactive aminofunctions of nucleobases in nucleotide substrates. Several medium-size PS2-ODNs were prepared by this approach. Their identity and purity were confirmed by means of ^{31}P NMR, gel electrophoresis, and mass spectrometry.

2.5.4 Synthesis of PS2-ODNs *via* a phosphotriester approach. The required phosphotriester is obtained in one step from the 5′-OH group of the growing chain and the nucleoside 3′-phosphodiester units. In a manner similar to the phosphate triester approach for synthesizing natural ODN, the groups of Caruthers and Dahl have synthesized the PS2 dimer and PS2-ODNs by the phosphotriester method using different phosphorylating and/or condensing agents.[7,76–80] Caruthers' laboratory reported the dithymidine phosphorodithioate **53** in Scheme 15 was prepared by chemoselective activation of the oxygen atom at the phosphorus center of **52**. The major challenge in this approach was to identify proper condensing agents. In addition, it is quite difficult to avoid the formation of the small percentage of undesired phosphothioate compound **54**. Very similarly, Dahl's laboratory introduced a new *S*-protecting



Scheme 14 Synthesis of PS2-ODN *via* a dithiaphospholane approach. In this reaction, Pya = *N*-methylpyrolidin-2-ylidenyl.

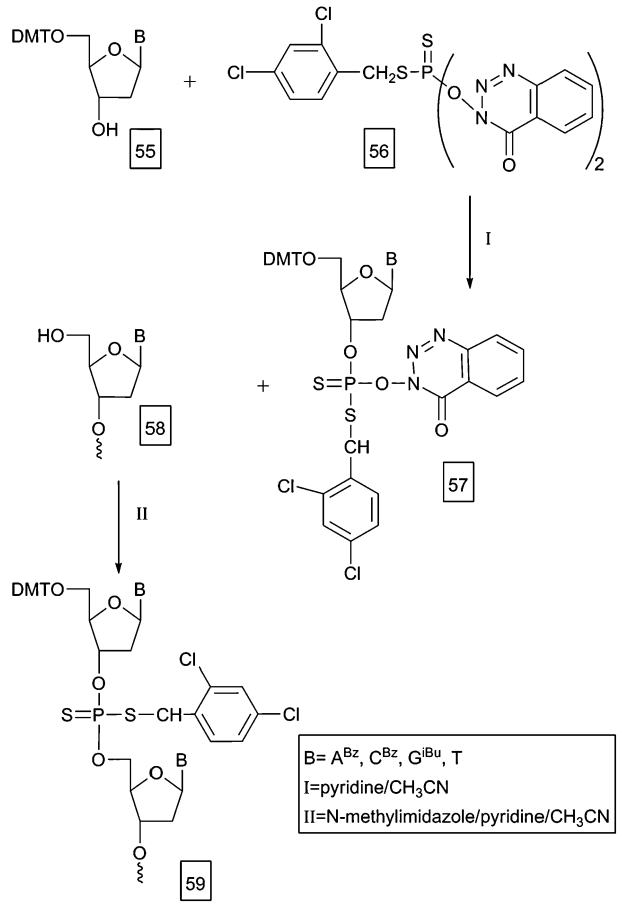


Scheme 15 Synthesis of dithymidine dithiophosphate *via* a phosphotriester approach.

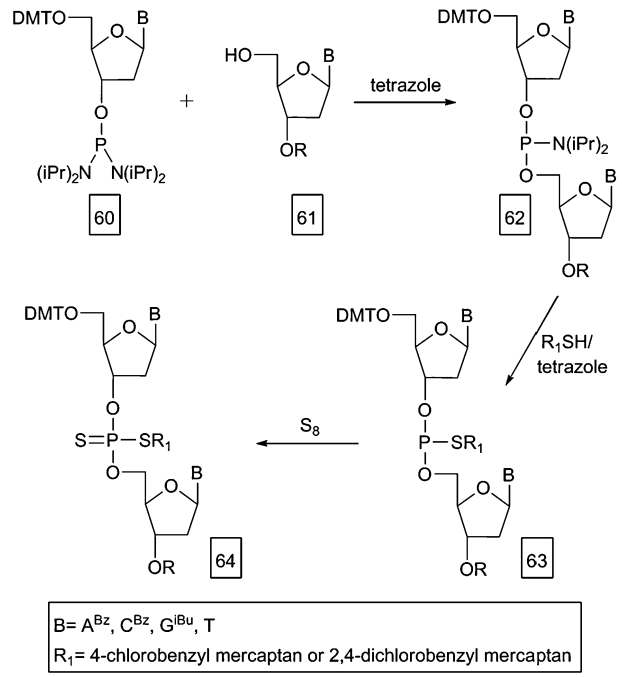
group to synthesize dithymidine dithiophosphate.[77] The undesired phosphothioate counterpart for an ODN will be challenging to remove during the PS2-ODN purification.

Dahl's laboratory also reported a modified triester method (a modified HOBt-method, HOBt is 1-hydroxybenzotriazole) for the preparation of PS2-ODNs containing all four bases.[81] As depicted in Scheme 16, the phosphorodithioate linkage is introduced by the reaction of a protected nucleoside **55** (or oligonucleotide) with a dithiophosphorylating agent **56** [RSP(S)(ODhbt)$_2$, R = 2,4-dichlorobenzyl, Dhbt = 3,4-dihydro-4-oxo-benzotriazin-3-yl] in pyridine and acetonitrile, followed by coupling the product **57** to a protected nucleoside **58** (or oligonucleotide) to form the dithiophosphotriester **59**. This method is suitable for large-scale synthesis and gives dithiophosphates without detectable phosphorothioate impurities by ^{31}P NMR. This method has been used to synthesize octamers containing PS2 linkages at all positions. The slow coupling step and limited stability of the dithiophosphorylating monomers make the method less attractive for automated syntheses. However, Dahl's laboratory improved the coupling rate by introducing two new dithiophosphorylating reagents, which have been shown to give nucleoside monomers with a reactivity suitable for solid phase synthesis.[78] Recently, Capaldi *et al.* have introduced a new dithiophosphorylating reagent DPSE-SP(S)Cl$_2$ (DPSE is 2-diphenylmethylsilylethyl) and have demonstrated the synthesis of a dimer and an ODN containing alternating dithiophosphate/phosphate diester linkages.[82]

2.5.5 Synthesis of PS2 dimers *via* a phosphorodiamidite approach. The nucleoside phosphorodiamidite approach (Scheme 17) was developed in Caruthers' laboratory.[83,84] Briefly, condensation of a nucleoside phosphorodiamidite **60** with 3′-protected nucleoside **61** in the presence of tetrazole



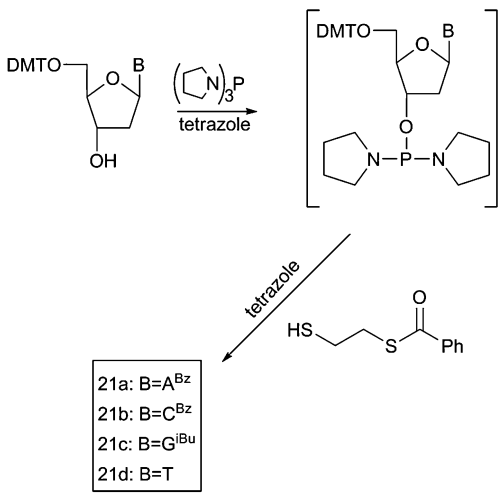
Scheme 16 Synthesis of PS2-ODN *via* a phosphotriester approach.



Scheme 17 Synthesis of PS2 dimer *via* a phosphorodiamidite approach.

yields dinucleoside phosphoramidite **62** which is then converted to the thiophosphite triester **63** by condensation with either 4-chlorobenzyl mercaptan or 2,4-dichlorobenzyl mercaptan using tetrazole or other activators as a catalyst. Sulfurization with elemental sulfur generates the completely protected dinucleoside dithiophosphate **64**. If **62** is sulfurized with H_2S in the presence of tetrazole, the *H*-phosphonothioate analog mentioned earlier is generated. It is possible to construct PS2-ODN in this way, but the procedure as a general method suffers from several potential limitations. This approach is more useful for its versatility to synthesize phosphorothioamidates and phosphorothioate triesters than for routine synthesis of PS2-ODN.

2.5.6 Synthesis of PS2-ODNs *via* a thiophosphoramidite approach. Synthesis of PS2-ODNs *via* thiophosphoramidite chemistry, independently introduced by Caruthers,[85–87] Gorenstein,[9,22–24] and Dahl,[88] is currently the most efficient method for preparing PS2-ODNs. Importantly, the thiophosphoramidites are commercially available from AM Biotechnologies (www.thioaptamer.com) after many years of Yang's efforts.[45] Since the initial reports on the synthesis of thiophosphoramidites, there have been numerous synthetic efforts directed toward the development of different sulfur protecting groups. The β-cyanoethyl protecting group has been used by Dahl's laboratory with limited success mainly due to high levels of phosphoromonothioate impurity (8–10%) contamination.[88,89] While the phosphoromonothioate contamination is reduced to more acceptable levels (2–4%) when the 2,4-dichlorobenzyl group is used to block sulfur,[85] deprotection of the resulting phosphorodithiotriester must be carried out by a post-synthesis treatment with either thiophenolate or an odorless substitute.[79] In addition, a thiophosphoramidite containing the 2,4-dichlorobenzyl group is relatively unstable to oxidation which necessitates changing the thiophosphoramidite solution on the DNA synthesizer every few hours. The various phosphorus protecting groups examined for the formation of PS2 linkage(s) led to the development of a novel base-labile β-(benzoylmercapto)ethyl protecting group (referred to as "Caruthers protecting group") as shown in compounds **21a–d** in Scheme 18. The Caruthers protecting group combines the best features of the two previously employed for synthesis of PS2-ODNs as it



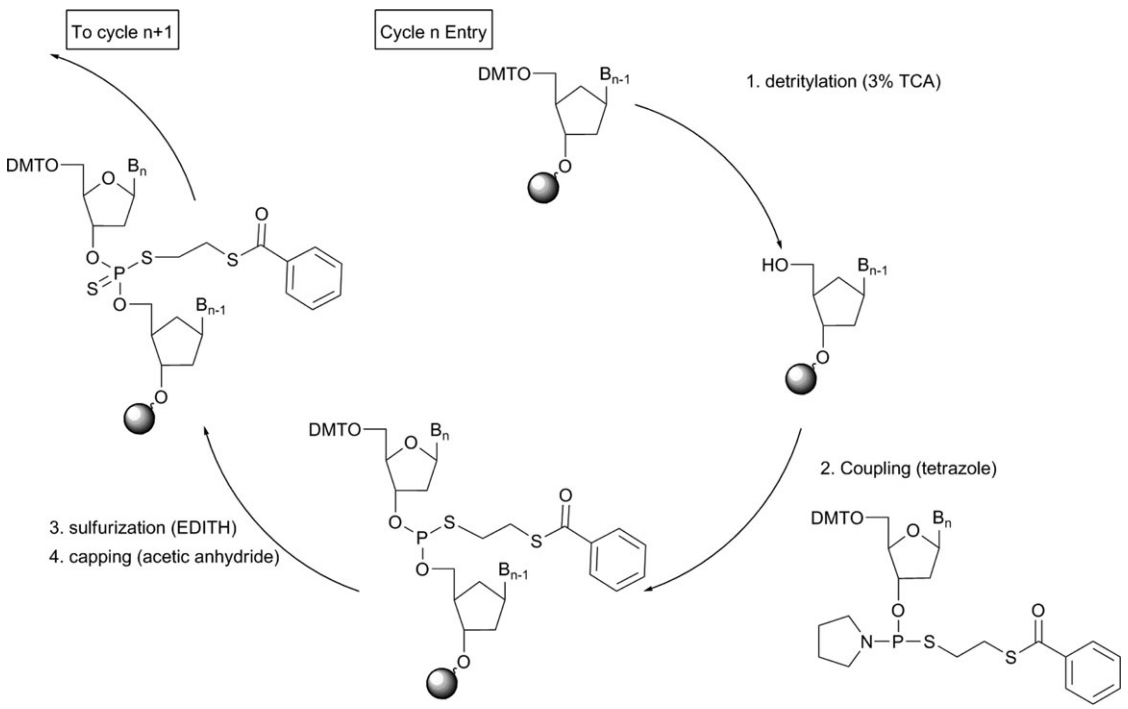
Scheme 18 Synthesis of commercially available thiophosphoramidites.

has a stability toward oxidation similar to thiophosphoramidites containing a β-cyanoethyl group and its removal can be conveniently carried out during a simple base-deprotection treatment to give the corresponding PS2-ODNs with relatively low (or non-detectable) levels of phosphormonothioate contamination.[65,87] The nucleoside 3′-thiophosphoramidites **21** were obtained from the appropriately protected nucleoside, tris(pyrrolidino)phosphine, and ethanedithiol monobenzoate *via* a one-flask synthesis procedure (Scheme 18).[87]

The solid-phase synthesis of PS2-ODNs is similar to conventional methods for synthesis of ODNs, but there are several essential modifications since two sulfur atoms are introduced into the phosphate linkage. The PS2-ODN synthesis cycle begins with the 3′-hydroxyl nucleoside attached to a solid controlled-pore glass (CPG) support or other support through a long spacer arm (Scheme 19). The first step in the synthesis is the removal of the DMT group with 3% TCA to free the 5′-hydroxyl for the coupling reaction. The next step, coupling, is achieved by simultaneously adding a thiophosphoramidite derivative of the next nucleotide and tetrazole (or other activators such as Activator-42). The tetrazole protonates the nitrogen of the thiophosphoramidite, making it susceptible to nucleophilic attack. This intermediate is very reactive and the following coupling step is completed in less than 5 min. The 5′-OH group of the thiophosphoramidite is blocked with the DMT group. The subsequent steps involved immediate sulfurization with DDTT or EDITH.[90] In contrast to the conventional order of oxidation after capping in the synthesis of standard oligonucleotides, sulfurization is more efficient and forms fewer by-products when carried out before capping. The capping step terminates any chains that did not undergo coupling. The unreacted chain has a free 5′-OH which can be terminated or capped by acetylation to become "failure products." This is achieved with acetic anhydride and 1-methylimidazole. This approach for synthesizing PS2-ODNs is fully compatible with the procedures developed for preparing normal ODNs, as well as phosphorothioate and methylphosphonate oligonucleotides based on phosphoramidite chemistry. Several ODNs were prepared that contained the PS2 linkages in combination with other types of internucleotide linkages such as methylphosphonate linkages. Thiophosphoramidite chemistry is the dominant method to synthesize PS2-ODN analogs.

3. Purification of PS2-ODN

Current synthetic approaches to PS2-ODNs involve building an ODN chain that is anchored to a solid support, through its 3′-OH group, and is elongated by coupling to its 5′-OH (Scheme 19). The yield of each of the chemistry steps in a given chain-elongation cycle will generally be less than 99%. Specifically, the step coupling yield and sulfurization yield are lower than that of the normal ODN synthesis. In addition, sulfur loss during deprotection steps (both concentrated ammonium and/or 80% acetic acid for the final DMT removal) is prone to increase the phosphorothioate and phosphate impurities. Moreover, since the sulfur substitutes interact strongly with certain metal cations and/or column materials, the quality of the deionized water and the column used in these procedures are very important.[89,91] Furthermore, ODNs exclusively containing PS2 linkages cannot be fully dissolved in water. All of these reasons make it difficult to obtain highly pure PS2-ODNs for biological applications. Denaturing polyacrylamide gel electrophoresis and reverse phase columns, especially Hamilton PRP-1 reverse phase columns, have been widely used for purification of "DMT OFF" and/or "DMT



Scheme 19 A cycle of solid-phase synthesis of PS2-ODN.

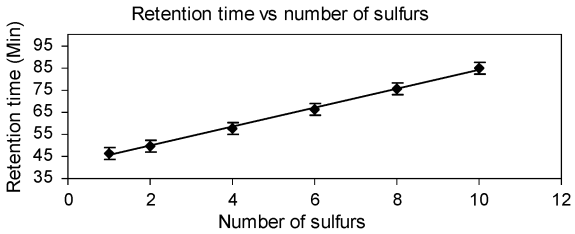


Fig. 5 Linearity of retention time by IEC with number of sulfurs. The resulting retention time is the average of the retention times of 14-mer sequences containing the same number of sulfurs. Column: Mono Q 10/10. Error bars are set at the maximum value ±2.7 min for NaCl.

ON'' PS2-ODNs exclusively containing PS2 linkages.[69,89,91] ODNs containing small percentages of dithioate linkages have been purified by ion-exchange chromatography (IEC).[16,65,92] We have developed a new and effective method of IEC separation of synthetic PS2 analogs from phosphorothioate contaminants by anion-exchange chromatography on a Mono Q column. Moreover, we have found that the retention time of a defined sequence PS2-ODN analog was sulfur dependent when the mobile-phase anion is either a chloride (Fig. 5 for example) or thiocyanate.[92] Using our method, phosphorothioate analog impurities are not detectable by ^{31}P NMR.[92]

4. Synthesis and screening of PS2-ODN library

4.1 Synthesis of PS2-ODN library

The success of *in vitro* thioaptamer selection[93–101] in combinatorial nucleic acid chemistry followed directly from studies showing that Sp-diastereomers of deoxynucleoside 5′-*O*-(1-thiotriphosphate)s (dNTP(αS)) are substrates for DNA polymerases and that NTP(αS) are substrates for RNA polymerases. Both types of enzyme catalyze formation of the phosphorothioate diester with an Rp-diastereomeric configuration.[6] The resulting thioaptamers with an Rp-diastereomeric configuration were nuclease resistant. However, the resistance of phosphorothioates to degradation by enzymes is weak. Polynucleotides containing phosphorothioate internucleotidic linkages were found to be broken down by nucleases only slower than the natural congeners.[102] Experiments have demonstrated that snake venom phosphodiesterase can cleave the selected Rp-thioaptamer diastereomer *in vitro*. Therefore, there is a need to select an even stronger nuclease resistant thioaptamer such as PS2-ODN. However, it is not possible to select strong nuclease-resistant achiral PS2-ODN analogs *via* the enzymatic *in vitro* selection method since dNTP(αS_2) and NTP(αS_2) are not substrates of polymerases.[17]

ODNs possessing multiple PS2 linkages appear to be "stickier" toward proteins than normal phosphate esters, attributable to nonspecific interactions. In order to optimize the total number of PS2 linkages, we have developed the split synthesis approach (Fig. 6) to construct PS2-ODN bead libraries.[16,103]

This approach can also create PS2-ODN libraries that only differ in the position of phosphate or dithioate but not in its base sequence. The positions of dithiophosphates in a mixed backbone PS2-ODN sequence can be determined by the reaction of the PS2-ODN with iodoethanol followed by

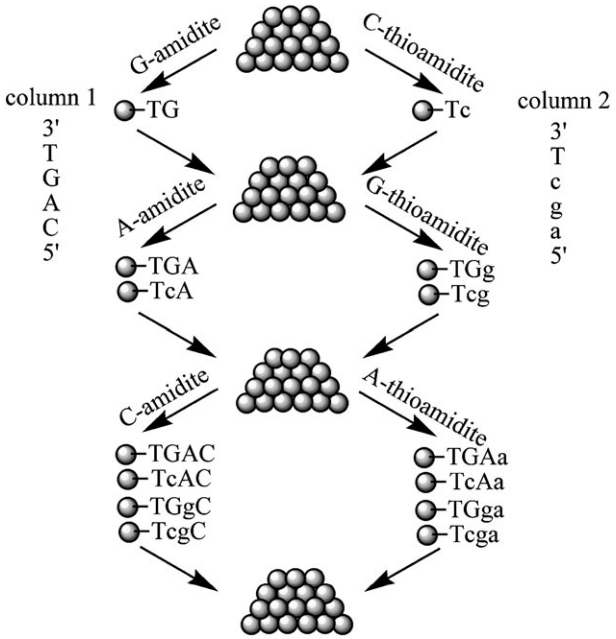


Fig. 6 General flow diagram for the solid-phase synthesis of a one-bead one-PS2-ODN library. Nucleoside dT was linked on solid support in advance. In the first cycle, in column 1, a phosphoramidite dG was used to form a dinucleotide phosphotriester dTG *via* a phosphotriester linkage, in column 2, a thiophosphoramidite C was used to form a dinucleotide dithiophosphotriester dTc *via* a phosphodithiotriester linkage. Upon pooling, the end products are a mixture of two kinds of bead-bound dinucleotides comprised of phosphorotriester and phosphodithiotriester. After splitting and pooling through three such cycles the eight (2^3) possible ODN and/or S2-ODN tetraoligonucleotides are represented on separate beads. A lower-case letter denotes a 3′-dithioate, while an upper-case letter denotes a 3′-phosphate.

base catalyzed cleavage of the thiophosphate triester.[104] The advantage of bead-based split-pool synthesis can be extended to as many other modifications as possible such as peptide nucleic acid *etc*.

By the split-pool method, two columns can create 2^N different members of the library for N split-pool steps. Utilizing more columns (M) allows the synthesis of M^N different beads with one unique thioaptamer sequence on each bead. The limit to the size of the combinatory library is the number of steps (N) and the number of columns (M) and of course the total number of beads, which is generally in the range of 10^6–10^8 or more depending upon the size of the beads and the synthesizer columns. Generally, the number of beads used in the synthesis should exceed the number of the ODNs in the library, in order to obtain adequate representation of all sequences. A ratio of 10 beads per sequence is recommended. More recently, a bead library of libraries has been created, with each bead containing a library of any complexity that could in principle have library sizes comparable to those created by *in vitro* combinatorial selection methods by utilizing mixtures of phosphoramidites/thiophosphoramidites (up to six different species) at selected positions in a given synthesis step. Therefore, it is easy to create 10^7–10^8 beads with 10^6 combinatorial library members on each bead yielding a total diversity, in principle, of 10^{13}–10^{14}; the same as *in vitro* combinatorial selection libraries.

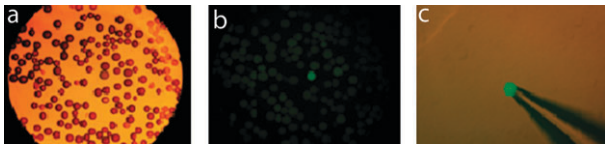


Fig. 7 A typical representative color micrograph of a bead-based dithioaptamer library screen. (a) An aliquot of PS2-ODN beads bound to NF-κB p50/p50 homodimer protein labeled with the Alexa Fluor 488 dye viewed under light microscopy. (b) The same beads viewed under fluorescence microscopy, in which a positive green bead stained with Alexa Fluor 488 dye can be easily identified in a background of many hundreds of nonreactive beads. (c) A single positive bead can easily be retrieved with a handheld micropipette under fluorescence microscopy.

4.2 Screening of the PS2-ODN library

A split-pool synthesis strategy for making PS2-ODN libraries is only the first step for bead-based thioaptamer selection. Since the point of making a library is to be able to rapidly identify a lead compound or set of lead compounds with desirable activities, the utility of any synthetic approach for making a library cannot be fully evaluated without knowing how screening and structure identification will be carried out. The filter binding method used in the enzymatic *in vitro* thioaptamer selection process is not suitable to identify a thioaptamer from a bead-based library. There are two developed assays to screen a bead-based combinatorial library. First, the bead library is exposed to a target covalently labeled with dye, then viewed under a fluorescence microscope. The positive beads are intensely stained when viewed by fluorescence and can be picked up with the aid of a micropipette coupled to a micromanipulator. This assay has been demonstrated in Fig. 7 by exposing a bead-based PS2-ODN library with an Alexa Fluor 488 dye labeled NF-kB to select thioaptamers.[103] The bead bound PS2-ODN library is directly exposed to a target, and adsorption is then assessed using a specific primary antibody and a second antibody conjugated with a fluorescent dye or with a fluorescently labeled antibody that binds to the complex.[105] Each individual positive bead selected from the PS2-ODN library was washed thoroughly with 8 M urea (pH 7.2) to remove the protein and was directly used for our "one-bead one-PCR" amplification using the 5′ and 3′ end primers.[103] The PCR product was cloned using the TA cloning procedure (Invitrogen) and sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

5. Properties of PS2-ODNs

5.1 Thermal stability of PS2-ODNs

The potential for a PS2-ODN to act as an antisense therapeutic tool lies in its ability to mediate high-affinity hybridization with the complementary RNA without the loss of sequence specificity. The duplex stability of a double-stranded nucleic acid is characterized by the melting temperature T_m, which is the temperature at which 50% of the double strand has dissociated into its two single strands. T_m depends on the concentration of the ODN, the properties of the solvent and the salt concentration. The duplex stability studies between an ODN containing PS2 linkages and a complementary unmodified DNA were carried out by Caruthers *et al*. and others. The result of these studies showed reduced melting temperatures for the modified duplexes in comparison to their unmodified counterparts with the degree of T_m depression proportional to the percentage of PS2 linkages on the ODN.[13,75,89] Caruthers *et al*. reported that oligomers containing less than 50% PS2 linkages form duplexes with melting temperatures depressed by 0.4–0.5 °C per modification whereas the duplexes generated from oligomers containing greater than 50% PS2 linkages have a T_m depression of 0.7 per linkage.[13] In addition, Caruthers *et al*. reported that PS2 linkages do not severely alter the shape of the T_m curve compared to phosphodiester linkages. Moreover, Caruthers *et al*. reported a graph of T_m *vs*. log[Na^+] that showed PS2-modified duplexes responded to increasing sodium ion in the same manner as a completely unmodified duplex DNA.[13] Bjergarde and Dahl have published somewhat greater ΔT_m values (0.8–2 °C) for some 17-mers containing PS2 linkages.[89] For fully substituted PS2-ODNs, duplex stability of a self-complementary 12-mer d(CGCGAATTCGCG) (Dickerson dodecamer) in which all phosphodiester linkages were replaced by PS2 linkages was studied using variable-temperature ^1H and ^{31}P NMR spectroscopy. Melting temperatures of the dodecamer, measured spectrophotometrically, showed significant decrease upon sulfur substitution (T_m 21 °C for the PS2 compared with 68 °C for the unmodified oligomer in 1 M salt).[106] These discrepancies may be due to different conditions of measurement and the sequences as well.

5.2 Nuclease resistance and serum stability

The nucleases (DNases, RNases) responsible for the degradation of nucleic acids (DNA, RNA) differ in their specificities. The most important nucleases are those specific for double-strand and single-strand *exo*- and endonucleases and the highly specific restriction endonucleases. The nucleases that degrade single strands, especially the exonucleases, are important in antisense ODNs. The nuclease resistance of PS2-ODNs was evaluated by several laboratories.[13,72,75,83,107] These studies indicated that PS2 linkages in ODNs are not hydrolyzed by nucleases. Caruthers *et al*. and Porritt and Reese have found that the PS2 linkage is entirely resistant to digestion by snake venom and calf spleen phosphodiesterase, nuclease P1, and the 3′-5′ exonuclease activity of T4-DNA polymerase.[72,108] However, the results of the resistance against DNase I were different from those of Ghosh *et al*. who claim that PS2-ODNs are as susceptible to DNase I as an unmodified ODN control.[109] In contrast, both Stec and Caruthers' laboratories demonstrated the stability of PS2-ODNs toward DNase I. In addition, Ghosh *et al*. reported that PS2-ODNs were no more stable than an unmodified ODN control in an MCF-7 cell nuclear fraction. The results of Ghosh *et al*. may reflect the ability of 5′-phosphorylated oligomers and oligomer analogs to act as substrates for phosphatase rather than indicate the susceptibility of the PS2 linkage to nuclease degradation.

5.3 Activation of RNase H mediated RNA cleavage

RNase H activation is considered to be one of the most important pathways for modulating gene expression. Among

many modified ODNs intended for use in antisense applications and capable of forming duplexes with RNA, only three phosphodiester analogs including phosphorothioate, PS2, and boranophosphate, combine hydrolytic stability and resistance to nucleases with the ability to efficiently elicit RNase H hydrolysis in RNA-DNA duplexes.[110]

In an early study by Ghosh *et al.*, a fully PS2 modified 17-mer ODN was designed to target the coding region of the rabbit β-globin mRNA and the degradation rate of the rabbit β-globin mRNA by both RNase H (*E. coli*) and RNase H1 (human) in the PS2-ODN-mRNA duplex.[109] It was found that the PS2-ODN produced slightly higher specific translation inhibition of rabbit β-globin mRNA compared to that of the unmodified ODN in a cell-free translation system. To assess the ability of PS2-ODNs to stimulate RNase H activity, a more comprehensive study was carried out by Caruthers' laboratory.[13] The ability of PS2-ODNs to direct RNase H degradation of U2 snRNA during an extended time in HaLa cell nuclear extract was examined. It was found ODNs containing up to 50% PS2 linkages were able to activate RNase H and direct RNase H degradation with the same efficiency as unmodified ODNs, while those containing from 50 to 100% PS2 linkages acted with somewhat reduced efficiency. At limiting concentrations, an ODN containing alternating PS2 and phosphate linkages was able to direct RNase H degradation of the target RNA in an extended incubation, while an unmodified ODN did not.[13] In a different study targeting the erbB-2 mRNA *via* antisense mechanism was examined in a breast cancer cell line with overexpresses erbB-2 oncogene.[111] A 15-mer *anti*-erbB-2 sequence synthesized as an alternating PS2/phosphodiester has shown to be effective in inhibiting the ebrB-2 mRNA expression and the production of erbB-2 protein.

5.4 Inhibition of human immunodeficiency virus activity[14,112]

During studies with PS2-ODNs as inhibitors of human immunodeficiency virus type 1 (HIV-1), Marshall *et al.* reported that PS2-linked deoxyoligocytidine analogs were very potent inhibitors of HIV-1 reverse transcriptase *in vitro*, as they exhibit an increasing inhibitory effect with length and number of PS2 linkages. In addition, Marshall *et al.* reported that the inhibitory effect can be at least 30-fold greater with PS2-linked oligodeoxycytidine than for the corresponding phosphorothioate analogs of similar length. In cell culture, PS2 linked oligodeoxycytidines were active inhibitors of syncytia formation and effectively inhibit *de novo* infection of target cells by HIV-1. More over, comparative experiments showed that a PS2-deoxycytidine 14-mer was as effective an inhibitor of *de novo* infection as a phosphorothioate-deoxycytidine 28-mer. Further more, a mechanism studied indicated that this type of inhibitor bound strongly to the primer-template active site of HIV-1 RT.[14]

5.5 Induction of B cell proliferation and differentiation

To investigate the role of the ODN backbone in determining the magnitude of the lymphocyto stimulation, Krieg *et al.* reported that PS2-ODNs, in which the central linkages are phosphodiesters but the two 5′ and five 3′ linkages are PS2 modified, were the most potent and induced B cell activation at nanomolar concentrations, approximately 1 log lower than required for the next most potent phosphorothioate modification.[15] However, they did not examine whether ODNs in which the entire backbone contains PS2-modifications may have even greater immune stimulation.

5.6 Interaction of PS2-ODNs with cellular proteins

The interaction of PS2-ODNs with certain cellular proteins deserves some attention. As the binding of PS2-ODNs to plasma proteins has been positively correlated to *in vivo* tissue distribution, the introduction of PS2 linkages is expected to improve the pharmacokinetic properties of PS2-ODNs by both increasing the stability toward degradation and improving the distribution to tissues. To investigate PS2-ODN binding to certain cellular proteins, Caruthers' laboratory used *E. coli* single-stranded DNA binding protein (SSB) as a model to examine its interactions with ODNs containing several different backbone chemistries including the PS2 modification and phosphorothioate modification and other modifications.[113] The PS2-ODN was found to bind to SSB with a higher affinity than the phosphordiester linkage. In addition, the length of the PS2-ODN was also an important factor in binding to SSB. A comparative study on this manner was examinated in Stein's laboratory.[64] Stein *et al.* have compared the cellular pharmacology of phosphorothioate and PS2-ODNs in HL60 cells, and evaluated their cell surface binding, internalization, and compartmentalization. In addition, the ability of phosphorothioate and PS2-ODNs to bind to rsCD4 and bFGF and to inhibit the activity of protein kinase C (PKC) was studied. These studies suggest that PS2-ODNs may have therapeutic potential in addition to their use as antisense agents.

5.7 Pharmacokinetic properties of PS2-ODN

To assess the pharmacokinetics and tissue distribution of PS2-ODNs, a fully PS2 modified 15-mer of a sequence complementary to the AUG region of K-*ras* were radiolabed with carbon-14. The PS2-ODN and control ODNs were administered as a single dose in the tail vein of nude mice harboring a K-*ras*-dependent human pancreatic tumor (CFPAC1). The kinetics of PS2-ODN availability in the bloodstream was followed. Concentration *versus* time profiles for the PS2-ODN and the control ODNs were biphasic, indicative of a two-compartment model. A rapid distribution phase with $t_{1/2}\alpha$ values of 1 min or less and an elimination phase with average $t_{1/2}\beta$ values of 24–35 min were observed. Value of distribution measurements (V_d) were 3.2 and 6.3 ml for PS2 and phosphorothioate, in comparison to 3.6 ml for sucrose, a fluid-phase marker. Relative tissue drug levels obtained at 1 and 24 h after administration were kidney > liver > spleen > tumor > muscle. Total kidney and liver PS2-ODN and control ODN accumulation was approximately 7–15% of the initial dose, with tumor accumulating 2–3%. Intact compound was recovered from all tissue, including tumor, as assessed by RP-HPLC coupled to radiometric detection. Integrity of the PS2-ODN and its control ODNs ranged from 73% in blood to 43–46% in kidney and liver. It was found that kidney and liver appeared to be the primary sites of metabolism. The widespread tissue availability of the PS2-ODN

suggests the use of the PS2-ODN as a potential antitumor agent.

5.8 Thioaptamer application

Thioaptamers offer advantages over traditional aptamers in their enhanced affinity and specificity and higher stability, largely due to the properties of the PS2 backbone-modifications. A novel bead-based thioaptamer selection protocol has been developed to produce potential thioaptamers targeting certain proteins.[103,114] We have demonstrated thioaptamers' potential and versatilities in diagnostic assay formats where they can capture very low abundance of proteins in crude cell extracts and biological toxins.[115] In addition, our nuclease-resistant thioaptamer XBY-S2 targeting AP-1 has been tested for antiviral activity against West Nile virus. Moreover, administration of XBY-S2 to Pichinde virus-infected guinea pigs resulted in a significant reduction in Pichinde virus-induced mortality.[95,97]

6. Conclusions

Nucleoside and oligonucleoside dithiophosphates are unique types of compounds in the family of phosphate-modified nucleic acids. They are the near or close mimics of natural nucleic acids. The hydrolytic stability and high nuclease resistance of the dithiophosphate linkage can be used for the creation of various biologically active conjugates and new classes of antiviral drugs. The increased lipophilicity of the dithiophosphate diesters may be employed in the delivery of dithiophosphate prodrugs to improve the antiviral activity of known chain terminating nucleoside analogs in the near future. The ability to direct RNase H degradation of the target RNA makes these analogs suitable candidates for antisense therapy. Their high affinity and high specific interactions with targeted proteins selected by combinatorial chemistry make them promising both as therapeutic lead compounds and agents for diagnostic applications. The commercial availability of the thiophosphoramidites is increasing research and development in various molecular biology applications and nucleic acid based diagnostics and therapeutics.

Acknowledgements

The authors are grateful for financial support from NIH grants (R03-CA121353-01, R43GM084552, and R43CA141842) to Xianbin Yang. Xianbin Yang wishes to express his sincere appreciation to Professor Wojciech Stec for his highly intellectual impact and every-day care during his stay in Poland.

References

1 P. C. Zamecnik and M. L. Stephenson, *Proc. Natl. Acad. Sci. U. S. A.*, 1978, **75**, 280–284.
2 M. L. Stephenson and P. C. Zamecnik, *Proc. Natl. Acad. Sci. U. S. A.*, 1978, **75**, 285–288.
3 A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818–822.
4 C. Tuerk and L. Gold, *Science*, 1990, **249**, 505–510.
5 F. Eckstein and G. Gish, *Trends Biochem. Sci.*, 1989, **14**, 97–100.
6 F. Eckstein, *Annu. Rev. Biochem.*, 1985, **54**, 367–402.
7 O. Dahl, *J. Sulfur Chem.*, 1991, **11**, 167–192.
8 J. Nielsen, W. K. D. Brill and M. H. Caruthers, *Tetrahedron Lett.*, 1988, **29**, 2911–2914.
9 N. Farschtschi and D. G. Gorenstein, *Tetrahedron Lett.*, 1988, **29**, 6843–6846.
10 M. Eto, *Organophosphorus Peptides*, CRC Press Inc., Cleveland, 1974.
11 J. Baraniak and W. J. Stec, *J. Chem. Soc., Perkin Trans. 1*, 1987, 1645–1987.
12 L. H. Parker-Botelho, L. C. Webster, J. D. Rothermel, J. Baraniak and W. J. Stec, *J. Biol. Chem.*, 1988, **263**, 5301–5305.
13 L. Cummins, D. Graff, G. Beaton, W. S. Marshall and M. H. Caruthers, *Biochemistry*, 1996, **35**, 8734–8741.
14 W. S. Marshall and M. H. Caruthers, *Science*, 1993, **259**, 1564–1570.
15 A. M. Krieg, S. Matson and E. Fisher, *Antisense Nucleic. Acid Drug Dev.*, 1996, **6**, 133–139.
16 X. Yang and D. G. Gorenstein, *Curr. Drug Targets*, 2004, **5**, 705–715.
17 J. Ludwig and F. Eckstein, *J. Org. Chem.*, 1991, **56**, 1777–1783.
18 J. Ludwig and F. Eckstein, *J. Org. Chem.*, 1991, **56**, 5860–5865.
19 I. Tworowska and W. Dabkowski, *Chem. Commun.*, 1998, 2611–2612.
20 K. E. Jenkins, A. P. Higson, P. H. Seeberger and M. H. Caruthers, *J. Am. Chem. Soc.*, 2002, **124**, 6584–6593.
21 C. L. Franklin, H. Li and S. F. Martin, *J. Org. Chem.*, 2003, **68**, 7298–7307.
22 Y. Cho, F. C. Zhu, B. A. Luxon and D. G. Gorenstein, *J. Biomol. Struct. Dyn.*, 1993, **11**, 685–702.
23 M. E. Piotto, J. N. Granger, Y. Cho and D. G. Gorenstein, *J. Am. Chem. Soc.*, 1990, **112**, 8632–8634.
24 D. G. Gorenstein, S. A. Schroeder, J. M. Fu, J. T. Metz, V. Roongta and C. R. Jones, *Biochemistry*, 1988, **27**, 7223–7237.
25 D. E. Volk, X. Yang, S. M. Fennewald, D. J. King, S. E. Bassett, S. Venkitachalam, N. Herzog, B. A. Luxon and D. G. Gorenstein, *Bioorg. Chem.*, 2002, **30**, 396–419.
26 P. Furrer, T. M. Billeci, A. Donati, C. Kojima, B. Karwowski, A. Sierzchala, W. Stec and T. L. James, *J. Mol. Biol.*, 1999, **285**, 1609–1622.
27 M. E. Piotto and J. N. Granger, *Tetrahedron*, 1991, **47**, 2449–2461.
28 M. E. Piotto, J. N. Granger, Y. Cho and D. G. Gorenstein, *J. Am. Chem. Soc.*, 1990, **112**, 8632–8634.
29 A. Okruszek, M. Olesiak, D. Krajewska and W. J. Stec, *J. Org. Chem.*, 1997, **62**, 2269–2272.
30 P. H. Seeberger, E. Yau and M. H. Caruthers, *J. Am. Chem. Soc.*, 1995, **117**, 1472–1478.
31 J. Jankowska, J. Sobkowska, J. Cieślak, J. Sobkowski, A. Kraszewski, J. Stawiński and D. Shugar, *J. Org. Chem.*, 1998, **63**, 8150–8156.
32 J. Jankowska, J. Cieslak, A. Kraszewski and J. Stawinski, *Tetrahedron Lett.*, 1997, **38**, 2007–2010.
33 T. Wada and T. Hata, *Tetrahedron Lett.*, 1990, **31**, 7461–7462.
34 M. Kalek, A. Bartoszewicz and J. Stawinski, *Nucleic Acids Symp. Ser.*, 2008, **52**, 285–286.
35 M. H. Caruthers, *Acc. Chem. Res.*, 1991, **24**, 278–284.
36 S. L. Beaucage, *Methods Mol. Biol.*, 1993, **20**, 33–61.
37 S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1992, **48**, 2223–2311.
38 P. H. Seeberger, P. N. Jorgensen, D. M. Bankaitis-Davis, G. Beaton and M. H. Caruthers, *J. Am. Chem. Soc.*, 1996, **118**, 9562–9566.
39 W. J. Stec, A. Grajkowski, M. Koziolkiewicz and B. Uznanski, *Nucleic Acids Res.*, 1991, **19**, 5883–5888.
40 W. J. Stec and A. Wilk, *Angew. Chem., Int. Ed. Engl.*, 1994, **33**, 709–722.
41 R. L. Letsinger and K. K. Ogilivie, *J. Am. Chem. Soc.*, 1969, **91**, 3350–3355.
42 J. Baddiley and A. R. Todd, *J. Chem. Soc.*, 1947, 648–651.
43 A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 1955, 2632–2638.
44 C. B. Reese and H. Yan, *Tetrahedron Lett.*, 2004, **45**, 2653–2656.
45 X. Yang, *Nucleic Acids Symp. Ser.*, 2008, **52**, 341.
46 B. Golos, J. M. Dzik, Z. Kazimierczuk, J. Ciesla, Z. Zielinski, J. Jankowska, A. Kraszewski, J. Stawinski, W. Rode and D. Shugar, *Biol. Chem.*, 2001, **382**, 1439–1445.
47 J. S. Richards, *Mol. Endocrinol.*, 2001, **15**, 209–218.
48 D. Waddleton, W. Wu, Y. Feng, C. Thompson, M. Wu, Y. P. Zhou, A. Howard, N. Thornberry, J. Li and J. A. Mancini, *Biochem. Pharmacol.*, 2008, **76**, 884–893.
49 J. Su, X. Yang, L. Wang and L. H. Zhang, *Acta Pharmaceutica Sinica*, 1996, **31**, 641–646.

50 N. Keppetipola and S. Shuman, *Nucleic Acids Res.*, 2007, **35**, 7721–7732.
51 Z. J. Lesnikowski, M. Jaworska and W. J. Stec, *Nucleic. Acids Symp. Ser.*, 1987, 273–276.
52 F. Kesbeke, J. Baraniak, R. Bulgakov, B. Jastorff, M. Morr, G. Petridis, W. J. Stec, F. Seela and P. J. Van Haastert, *Eur. J. Biochem.*, 1985, **151**, 179–186.
53 S. J. Scheinman, W. J. Stec and R. Coulson, *Mineral and Electrolyte Metabolism*, 1985, **11**, 85–90.
54 C. A. O'Brian, S. O. Roczniak, H. N. Bramson, J. Baraniak, W. J. Stec and E. T. Kaiser, *Biochemistry*, 1982, **21**, 4371–4376.
55 R. L. Jarvest, G. Lowe, J. Baraniak and W. J. Stec, *Biochem. J.*, 1982, **203**, 461–470.
56 R. J. de Wit, J. Hoppe, W. J. Stec, J. Baraniak and B. Jastorff, *Eur. J. Biochem.*, 1982, **122**, 95–99.
57 C. Erneux, D. Couchie, J. E. Dumont, J. Baraniak, W. J. Stec, E. G. Abbad, G. Petridis and B. Jastorff, *Eur. J. Biochem.*, 1981, **115**, 503–510.
58 P. M. Burgers, F. Eckstein, D. H. Hunneman, J. Baraniak, R. W. Kinas, K. Lesiak and W. J. Stec, *J. Biol. Chem.*, 1979, **254**, 9959–9961.
59 W. S. Zielinski and W. J. Stec, *J. Am. Chem. Soc.*, 1977, **99**, 8365–8366.
60 *REviews on Heteroatom Chemistry*, ed. J. Baraniak and W. J. Stec, Tokyo, 1993.
61 C. B. Reese, L. H. K. Shek and Z. Zhao, *J. Chem. Soc., Perkin Trans. 1*, 1995, 3077–3084.
62 F. Eckstein, *J. Am. Chem. Soc.*, 1970, **92**, 4718–4723.
63 M. Wenska, J. Jankowska, M. Sobkowski, J. Stawinski and A. Kraszewski, *Tetrahedron Lett.*, 2001, **42**, 8055–8058.
64 J. L. Tonkinson, M. Guvakova, Z. Khaled, J. Lee, L. Yakubov, W. S. Marshall, M. H. Caruthers and C. A. Stein, *Antisense Res. Dev.*, 1994, **4**, 269–278.
65 X. Yang, S. Fennewald, B. A. Luxon, J. Aronson, N. K. Herzog and D. G. Gorenstein, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 3357–3362.
66 G. M. Porritt and C. B. Reese, *Tetrahedron Lett.*, 1989, **30**, 4713–4716.
67 J. Stawinski, M. Thelin and R. Zain, *Tetrahedron Lett.*, 1989, **30**, 2157–2160.
68 C. E. Dreef, G. A. Dreef-Tromp, G. A. van der Marel and J. H. van Broom, *Synlett*, 1990, 481–483.
69 K. Kamaike, K. Hirose, Y. Kayama and E. Kawashima, *Tetrahedron Lett.*, 2004, **45**, 5803–5806.
70 W. K. D. Brill, E. Yau and M. H. Caruthers, *Tetrahedron Lett.*, 1989, **30**, 6621–6624.
71 P. H. Seeberger and M. H. Caruthers, *Tetrahedron Lett.*, 1995, **36**, 695–698.
72 G. M. Porritt and C. B. Reese, *Tetrahedron Lett.*, 1990, **31**, 1319–1322.
73 T. Murata, S. Iwai and E. Ohtsuka, *Nucleic Acids Res.*, 1990, **18**, 7279–7286.
74 A. Okruszek and W. J. Stec, *Tetrahedron Lett.*, 1992, **33**, 7585–7588.
75 A. Okruszek, A. Sierzchala, K. L. Feraron and W. J. Stec, *J. Org. Chem.*, 1995, **60**, 6998–7005.
76 E. K. Yau, Y. X. Ma and M. H. Caruthers, *Tetrahedron Lett.*, 1990, **31**, 1953–1956.
77 J. Kehler, A. Puschl and O. Dahl, *Nucleosides, Nucleotides Nucleic Acids*, 1997, **16**, 23–32.
78 A. B. Eldrup, J. Felding, J. Kehler and O. Dahl, *Tetrahedron Lett.*, 1995, **36**, 6127–6130.
79 B. H. Dahl, K. Bjergarde, J. Nielsen and B. H. Dahl, *Tetrahedron Lett.*, 1990, **31**, 3489–3492.
80 B. H. Dahl, K. Bjergarde, V. B. Sommer and O. Dahi, *Acta Chem. Scand.*, 1989, **43**, 896–901.
81 A. B. Eldrup, K. Bjergarde, J. Felding, J. Kehler and O. Dahl, *Nucleic Acids Res.*, 1994, **22**, 1797–1804.
82 D. C. Capaldi, D. L. Cole and V. T. Ravikumar, *Nucleic Acids Res.*, 2000, **28**, 40e.
83 A. Grandas, W. S. Marshall, J. Nielsen and M. H. Caruthers, *Tetrahedron Lett.*, 1989, **30**, 543–546.
84 W. K. D. Brill, J. Nielsen and M. H. Caruthers, *J. Am. Chem. Soc.*, 1991, **113**, 3972–3980.
85 W. K. D. Brill, J. Y. Tang, Y. X. Ma and M. H. Caruthers, *J. Am. Chem. Soc.*, 1989, **111**, 2321–2322.
86 W. K. D. Brill, J. Nielsen and M. H. Caruthers, *Tetrahedron Lett.*, 1988, **29**, 5517–5520.
87 W. T. Wiesler and M. H. Caruthers, *J. Org. Chem.*, 1996, **61**, 4272–4281.
88 B. H. Dahl, K. Bjergarde, V. B. Sommer and O. Dahl, *Acta Chem. Scand.*, 1989, **43**, 896–901.
89 K. Bjergårde and O. Dahl, *Nucleic Acids Res.*, 1991, **19**, 5843–5850.
90 X. Yang, unpublished work, 2009.
91 W. T. Wiesler, W. S. Marshall and M. H. Caruthers, *Methods Mol. Biol.*, 1993, **20**, 191–206.
92 X. Yang, R. P. Hodge, B. A. Luxon, R. Shope and D. G. Gorenstein, *Anal. Biochem.*, 2002, **306**, 92–99.
93 J. Kang, M. S. Lee, J. A. Copland, 3rd, B. A. Luxon and D. G. Gorenstein, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 1835–1839.
94 J. Kang, M. S. Lee, S. J. Watowich and D. G. Gorenstein, *FEBS Lett.*, 2007, **581**, 2497–2502.
95 S. M. Fennewald, E. P. Scott, L. Zhang, X. Yang, J. F. Aronson, D. G. Gorenstein, B. A. Luxon, R. E. Shope, D. W. Beasley, A. D. Barrett and N. K. Herzog, *J. Gen. Virol.*, 2007, **88**, 981–990.
96 V. Thiviyanathan, A. D. Somasunderam and D. G. Gorenstein, *Biochem. Soc. Trans.*, 2007, **35**, 50–52.
97 X. Yang, H. Wang, D. W. Beasley, D. E. Volk, X. Zhao, B. A. Luxon, L. O. Lomas, N. K. Herzog, J. F. Aronson, A. D. Barrett, J. F. Leary and D. G. Gorenstein, *Ann. N. Y. Acad. Sci.*, 2006, **1082**, 116–119.
98 A. Somasunderam, M. R. Ferguson, D. R. Rojo, V. Thiviyanathan, X. Li, W. A. O'Brien and D. G. Gorenstein, *Biochemistry*, 2005, **44**, 10388–10395.
99 S. E. Bassett, S. M. Fennewald, D. J. King, X. Li, N. K. Herzog, R. Shope, J. F. Aronson, B. A. Luxon and D. G. Gorenstein, *Biochemistry*, 2004, **43**, 9105–9115.
100 D. J. King, S. E. Bassett, X. Li, S. A. Fennewald, N. K. Herzog, B. A. Luxon, R. Shope and D. G. Gorenstein, *Biochemistry*, 2002, **41**, 9696–9706.
101 D. J. King, D. A. Ventura, A. R. Brasier and D. G. Gorenstein, *Biochemistry*, 1998, **37**, 16489–16493.
102 B. Nawrot, N. Paul, B. Rebowska and W. J. Stec, *Mol. Biotechnol.*, 2008, **40**, 119–126.
103 X. Yang, S. E. Bassett, X. Li, B. A. Luxon, N. K. Herzog, R. E. Shope, J. Aronson, T. W. Prow, J. F. Leary, R. Kirby, A. D. Ellington and D. G. Gorenstein, *Nucleic Acids Res.*, 2002, **30**, 132e.
104 G. Gish and F. Eckstein, *Science*, 1988, **240**, 1520–1522.
105 Y. Yang, M. Yi, D. J. Evans, P. Simmonds and S. M. Lemon, *J. Virol.*, 2008, **82**, 10118–10128.
106 J. W. Jaroszewski, V. Clausen, J. S. Cohen and O. Dahl, *Nucleic Acids Res.*, 1996, **24**, 829–834.
107 O. Sakatsume, H. Tanaka and H. Takaku, *Nucleic Acids Symp. Ser.*, 1992, 195–196.
108 M. H. Caruthers, G. Beaton, L. Cummins, D. Graff, Y. X. Ma, W. S. Marshall, H. Sasmor, P. Norris and E. K. Yau, *Ciba Found Symp.*, 1991, **158**, 158–166; discussion 166-158.
109 M. K. Ghosh, K. Ghosh, O. Dahl and J. S. Cohen, *Nucleic Acids Res.*, 1993, **21**, 5761–5766.
110 J. Micklefield, *Curr. Med. Chem.*, 2001, **8**, 1157–1179.
111 J. P. Vaughn, J. Stekler, S. Demirdji, J. K. Mills, M. H. Caruthers, J. D. Iglehart and J. R. Marks, *Nucleic Acids Res.*, 1996, **24**, 4558–4564.
112 W. S. Marshall, G. Beaton, C. A. Stein, M. Matsukura and M. H. Caruthers, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 6265–6269.
113 X. Cheng, R. K. DeLong, E. Wickstrom, M. Kligshteyn, S. H. Demirdji, M. H. Caruthers and R. L. Juliano, *J. Mol. Recognit.*, 1997, **10**, 101–107.
114 X. Yang and D. G. Gorenstein, *Curr. Drug Targets*, 2004, **5**, 705–715.
115 H. Wang, X. Yang, G. C. Bowick, N. K. Herzog, B. A. Luxon, L. O. Lomas and D. G. Gorenstein, *Biochem. Biophys. Res. Commun.*, 2006, **347**, 586–593.