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SYNTHESIS, NMR AND STRUCTURE OF OLIGONUCLEOTIDE PHOSPHORODITHIOATES

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Abstract Thiophosphoramidite as well as thiophosphoramidate chemistry has been used to prepare dithiophosphate analogues of oligonucleotides. The ^{1}H and ^{31}P resonances of the 3'-thymidine phosphorodithioate decamer d(CGCTpS_2^-TpS_2^-AAGCG) were assigned by two-dimensional NMR and the solution structure determined.

Introduction

Oligonucleotides and various analogues of oligonucleotides are known to exhibit antiviral activity. Thus addition of complementary "antisense" oligodeoxynucleotides to cells in culture has been found to specifically inhibit expression of a number of genes.[1] Oligothymidylate, polyribonucleotides, thiono-polycytidylate, oligodeoxyribomethylphosphonate nucleosides have all been shown to have antiviral activity.[2] Zamecnik[3], Matsukura[4] and Wickstrom[5] have shown that oligodeoxyribonucleotides have anti-HIV activity. Oligodeoxynucleotides, however, are susceptible to nuclease digestion[5] and are not stable enough for intravenous or oral administration.

Oligodeoxynucleoside methylphosphonate and phosphorothioate[4] analogues are nuclease resistant, enter animal cells and protect them from viral challenge, but the diastereomeric phosphonate mixtures (due to the new chiral phosphorus center) are unexpectedly less effective *in vitro* than normal oligodeoxynucleotides, apparently requiring high concentrations (100-300 μM) for effective antiviral activity. Similar difficulty exists with the phosphorothioates It is believed that diastereomerically pure phosphonate or phosphorothioate analogues could be much more effective than mixtures.[6]

Recently Caruthers, Gorenstein and coworkers synthesized phosphorodithioate oligonucleotide analogues[7-9] which have been shown to be nuclease resistant and are 36–600 times more effective in inhibiting HIV reverse transcriptase than the normal antisense oligonucleotide or the monothio analogue.[10] Importantly, in contrast to the monothiophosphate and methylphosphonate oligonucleotide analogues, the phosphorus center in the phosphorodithioates is achiral and hence problems associated with diastereomeric mixtures are avoided. The phosphorodithioates thus represent a potentially useful class of oligonucleotide analogues However, no information is available on the structural perturbations created by the phosphorodithioate moiety in oligonucleotides

Most reported syntheses of a dithiophosphate analogues of oligonucleotide have used thiophosphoramidite chemistry[7-9,11-14] Although H-phosphonate[15-18] and dithiophosphate triester[19] chemistries have also been described, there have been no previous reports of P(V) phosphoramidate chemistry in the synthesis of dithiophosphate analogues In this paper we describe the synthesis of dithiophosphate analogues of DNA using both deoxynucleoside 3'-aryl thiophosphoranilidate and solution and solid phase thiophosphoramidite chemistries The synthesis and purification of a dithiophosphate analogue of an oligonucleotide at the 10 μmole level using the phosphoramidite chemistry is also reported. The complete assignment of the ^{1}H and ^{31}P spectra of a dithioate analogue,

the decamer d(CGCTpS$_2^-$TpS$_2^-$AAGCG) (pS$_2$- represents a phosphorodithioate replacing the 3'-thymidine phosphate, identified as the TpS$_2^-$-decamer) has been made. Using a hybrid matrix/restrained molecular dynamics refinement procedure we have determined the structure of the modified oligonucleotide in solution. In contrast to the parent phosphoryl decamer the dithiophosphate analogue exists as a hairpin loop at low salt in solution.

Experimental

Synthesis of Phosphorodithioates via Thiophosphoramidite Chemistry

Dichloro-N,N-diisopropylaminophosphine, 1, was synthesized by the reaction of phosphorus trichloride with diisopropylamine (2 equiv).[20] Distillation (56-59 °C/0.5 mm Hg) afforded pure phosphine as shown by ^{31}P NMR (δ ppm in benzene-d_6, downfield relative to external 85% H_3PO_4· 169.0[20]).

Chloro-N,N-diisopropylaminothiomethoxyphosphine, 2. A 50 ml addition funnel was charged with a suspension of sodium thiomethoxide (22.8 mmol) and catalytic amount (2 28 mmol) of KI in 40 ml of anhydrous dichloromethane. This suspension was added dropwise over a period of 10 h at −65 °C to a stirred solution of dichloro-N,N-diisopropylaminophosphine, 1 (24.7 mmol plus a catalytic amount of $AlCl_3$ (0.5 mmol)) in 20 ml of anhydrous CH_2Cl_2. Generally, after addition of sodium thiomethoxide the resulting suspension was allowed to stir for 3 h at −35 °C, then 5 h at −20 °C before stirring for a final 10 h at r.t. The suspension was then vacuum filtered and the sodium chloride salt was washed with 100 ml anhydrous ether. The filtrate was evaporated under a dry nitrogen atmosphere and reduced pressure (100 mm Hg) at room temperature. The purity of the crude residue was checked by ^{1}H and ^{31}P NMR (in benzene-d_6 solvent: chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, δ ^{31}P 168.0 ppm; N,N-diisopropylaminodithiomethoxyphosphine impurity, 3, δ 118.2 ppm). The chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, is relatively unstable and partially decomposes during purification by distillation, requiring high vacuum (b.p , 55-60 °C/0.05 mm Hg). The above synthetic route gave ca 95% of the desired product 2 and less than 5% of 3 and was therefore used for further reaction without purification.

Preparation of O-(5'-O-(dimethoxytritylthymidin-3'-yl) S-methyl N,N-diisopropylthiophosphoramidite, 4. Excess, very reactive, 2 (5.52 mmol) was added to a mixture of diisopropylethylamine (7 4 mmol), 5'-O-(dimethoxytrityl protected thymidine (1.84 mmol) in 4 ml dichloromethane at r t The complete reaction course can be monitored by tlc (silica gel) and by ^{31}P NMR spectroscopy.

After completion of the reaction the mixture was transferred to a separatory funnel and diluted with ethyl acetate. The solution was washed with a saturated $NaHCO_3$ aqueous solution (25 mL × 3). This was followed by an additional washing with saturated NaCl aqueous solution (25 mL × 3). This was done under nitrogen at 4 °C within 15 minutes. The organic layer was dried over magnesium sulfate overnight and the solvent was evaporated to a foam under reduced pressure. The residue was then taken up in a few ml of dichloromethane and precipitated in 500 ml of ether-n-hexane mixture (−78 °C). The crude suspension is > 80% pure O-(5'-O-(dimethoxytritylthymidin-3'-yl) S-methyl N,N-diisopropylthiophosphoramidite, 4 (55 45 diastereomeric mixture – the ^{31}P NMR spectrum of 4 shows two signals at 164 85 and 163 14 ppm corresponding to a diastereomeric mixture of the thiophosphoramidite). The CI (70ev) MS of 4 shows a prominant pseudo-molecular ion peak at m/z 722 $(M+H)^+$. Additional intense ions are also observed at m/z 692 $(M\text{-}2\times CH_3 + H)^+$, 674 $(M - SCH_3)^+$ and 596 $(M-1 - \text{thyminyl})^+$ ^{1}H NMR (benzene-d_6, δ ppm) 1.0 (12H, d, $(CH_3)_2CH)_2N$), 1.55 (3H, s, thymine-CH_3), 2.27 (3H, d, CH_3S), 3 30 (6H, s, CH_3O), 3.52 (2H, m, 5',5"), 4.20 (1H, br. s, H4'), 4.78 (1H, br s, H3'), 6.50 (1H, br. m, H1'), 7.20 (13H, m, aromatic)

In addition to the major peaks assigned to 4, there are some minor ^{31}P peaks at 146.3 ppm and 13.2 ppm, which are assigned to the 3'-3' nucleoside dimer and a phosphoamidous acid, respectively (^{31}P chemical shift for phosphoamidous acids. 13.1 ppm) [21]

Synthesis of O-(5'-O-dimethoxytritylthymidin-3'-yl) S-(2,4-dichlorobenzyl) N,N-dimethyl thiophosphoramidites, 4 Syntheses of A, G, C and T S-2,4-dichlorobenzyl thiophosphoramidites (4, R' = 2,4-dichlorobenzyl; R = methyl) were as reported in ref. 7. To investigate the quality of the coupling of the thiophosphoramidites during oligonucleotide synthesis, high purity thymidine 3'-thiophosphoramidite was isolated by chromatographic separation. After evaporation of the crude, thymidine 3'-S-(2,4-dichlorobenzyl) thiophosphoramidite to a gum, 3 ml of CH_2Cl_2 ethylacetate: TEA (47.5.47 5 5 0) was used to dissolve 0.5 mmol of the crude product. Flash chromatography using a column of silica gel (320-400 mesh) 5 cm x 1 cm, with the above solvent as eluent, evaporation of the solvent under a stream of nitrogen allowed the pure product in a 70-80% yield, purity 100% by ^{31}P NMR δ_2 172.3, 170 6 ppm, lit.[7] 172.1, 170.4). We have also found that increased yields of the purified thiophosphoramidites are obtained by running the syntheses and column purification in an inert atmosphere dry box

Preparation of O-(3'-O-t-butyldimethylsilylthymidin-5'-yl) O-(5'-dimethoxytritylthymidin-3'-yl) phosphorodithioate, 7 3'-O-t-butyldimethylsilylthymidine was prepared as previously described [22] Addition of ca molar equivalent (0 042 mmole) of the protected nucleoside to 0.046 mmol of thiomethylphosphoramidite, 4 (R' = Me, R = i-Pr) in a benzene/acetonitrile solution (1·3) at r t followed by addition of 1H-tetrazole (0 8 mmol) resulted in the complete disappearance over several hours of the two ^{31}P signals at 164 9 and 163 1 ppm, which are replaced by two new signals at 190.7 and 191.3 ppm (55.45 ratio), which are assigned to a R_P and S_P diastereomeric mixture of O-(3'-O-t-butyldimethylsilylthymidin-5'-yl O-(5'-dimethoxytritylthymidin-3'-yl) S-methyl thiophosphite, 5 The best yield of the thiophosphite, 5, was obtained by reaction for 60 m

After sulfurization of 5 with a large excess of sulfur in pyridine the 3',5'-dithymidine methyl phosphorodithioate, 6 (R' = Me) were observed as two ^{31}P signals at 95.9 and 95.4 ppm (benzene-d_6). The sulfurization reaction was rapid, and after 10 min. at ambient temperature ca. 5% of the unreacted dinucleoside thiophosphite was still present. Similar results were obtained for the 3'-O-acetyl protected thymidine. The ^{31}P signals of the acetyl protected dithioate triester appear at 96.8 and 97.2 ppm (benzene-d_6).

S-demethylation of 3',5'-dithymidine methyl phosphorodithioate was accomplished by reaction with thiophenol/diisopropylethylamine for 40 h at rt to yield the achiral 3',5'-dithymidine phosphorodithioate, 7 (^{31}P, 114 9 ppm).

Solid Phase Syntheses of Dithiophosphate Modified Decamers

Syntheses of the dithiophosphate decamer (10 μmole) followed a manual modification[23,24] of the solid phase support methodology[7,25] using either deoxynucleoside 3'-O-(methyl) phosphoramidites (from Beckman) or the thiophosphoramidites, 4 (R' = 2,4-dichlorobenzyl, R = Me). All thiophosphoramidites were double coupled A 10-fold excess of the thiophosphoramidite, 4 was dissolved in acetonitrile and mixed with 50-fold excess of tetrazole.[9] The reaction time was ca. 10 min/cycle. For incorporation of the dithiophosphates, the oxidation step with iodine was replaced with sulfur (1.8 ml 5% elemental sulfur in CS_2/2, 6-lutidine (1:1) for 6 min, and repeated 4 times). The deprotection step following synthesis was as described[7]: 20 h in 2 ml of a thiophenol solution and 24 h in ammonium hydroxide. The oligonucleotides were purified by C-18 reverse phase HPLC with an acetonitrile/triethylammonium acetate (TEAA) gradient on a semi-prep Econosil C18 (Alltech) column. The TEAA buffer was a 0.1 M solution at pH 7.2. The sample was detritylated with 75% acetic acid for 10 minutes at room temperature followed by extraction with ether (5 times). The decamer sample was then treated for 10 min with Chelex-100, 200-400 mesh, with repeated vortexing. After repeated lyophilization from D_2O, the NMR sample was dissolved in a total volume of 600 μl of 99.96% D_2O.

The large downfield ^{31}P shift (ca. 110 ppm) of the dithiophosphate groups relative to the phosphate moiety (ca. -4 ppm) provides a convenient monitor of the success of the dithiophosphate analogue synthesis. The two dithiophosphoryl substitutions into the decamer show reduction in the number of phosphoryl-oligonucleotide signals with replacement by two new dithiophosphoryl signals.

Synthesis of Phosphorodithioates via Thiophosphoranilidate Chemistry

Phenyl-N-thiophosphoramido dichloride, 9. Treatment of thiophosphoryl chloride, 8 (135.2 mmol) with a mixture of aniline (135.2 mmol) and 27 ml 20% sodium hydroxide in H_2O at -5 °C for 1 h followed by addition of benzene and stirring for several additional hours at 5 °C gave a crude white slurry.

The resulting mixture was transferred to a separatory funnel and diluted with 200 ml benzene followed by washing with saturated aqueous NaCl which contained 1% sodium hydrogen sulfate (100 ml 3 ×). The organic layer was dried over magnesium and sodium sulfate (50:50 w/w) overnight. The benzene was removed under vacuum and the liquid residue was fractionally distilled. The first fraction was the desired product 9 (72% yield; b.p., 90-95 C/0.5 mm Hg; colorless liq). Unreacted thiophosphoryl chloride was collected in the liquid nitrogen trap. ^{31}P NMR of 9 shows one signal at 47.42 ppm ($CDCl_3$); ^1H NMR (benzene-d_6), δ 6.5-7 0 ppm, multiplet.

Synthesis of O-(5'-O dimethyoxytritylthymidin-3'-yl) O-(4-nitrophenyl) thiophosphoroanilidate, 11

5'-Dimethoxytritylthymidine (1.84 mmol) was treated in pyridine solution with 5-fold molar excess of phenyl-N-thiophosphoramido dichloride 9 (9.3 mmol) at 25°C for 2 h to give the reactive 2'-deoxyribonucleoside-3'-chlorothiophosphoranilidate 10, which, without isolation, was condensed with p-nitrophenol (18.0 mmol) (25°C) in pyridine to yield the crude 3'-aryl thiophosphoranilidate, 11. After 2 h the reaction was quenched with an excess of H_2O (20 ml) The reaction mixture was treated with benzene and NaCl-H_2O (200 ml). After aqueous workup, which removes the pyridinium salt, excess p-nitrophenol and all hydrolysis products of 9, the organic phase was separated and evaporated to dryness. The oily residue was further coevaporated with toluene (2 x 10 ml). The crude 11 was partially dissolved in benzene (100 ml). The benzene layer was dried for 24 h over $MgSO_4$. The benzene solution was concentrated to 3 ml and this residue was redissolved in CH_2Cl_2 (10 ml), and this solution was added (at -20°C) dropwise into n-pentane to afford the thiophosphoroanilidate, 11.

The thiophosphoroanilidate 11 was filtered off, washed and dried under an argon atmosphere in 70% isolated yield ^{31}P NMR (C_6D_6) for 11, δ = 52.90, 53.15 ppm for the Sp/Rp-isomers in a 55:45 ratio. The ^{31}P NMR chemical shift of the side product 14, isolated by means of combined-extraction comes at δ = 54.8 ppm (in C_6D_6 + CD_3CN, 1:1). ^1H NMR for 11 (benzene-d_6, ppm) 1.55 (3H, s, thymine-CH_3), 3.32 (6H, s, CH_3O), 4.30 (1H, dd,H4'), 6 40 (5H, sharp aniline aromatic), 7.00 (13H, br.m, aromatic), 7.85 (4H, dd., nitroaromatic). The FAB mass spectrum of 11 shows a prominant pseudo-molecular ion peak at m/z 837 $(M + H)^+$ Additional ions are also observed at m/z 678 (reflecting the loss of the thyminyl moiety and O of the nitro group from the molecular ion), and m/z 535 (M - DMT). A third, reasonably strong, ion in the mass spectrum, with an approximate abundance of 40% relative to the base ion (matrix: 3-nitrobenzyl alcohol, 100%) is detected at m/z 391 and arises from the loss of the thyminyl and DMT moiety from the molecular ion. The compound is stable if stored at -18°C.

Synthesis of O-(5'-O-t-butyldimethylsilylthymidin-3'-yl) O-(5'-dimethoxytritylthymidin-3'-yl) thiophosphoroanilidate, 12.

3'-O-t-butyldimethylsilylthymidine was condensed with nucleotide 11 by modification of the procedure of ref. 26 Thus to the final solution of the lithium derivative generated from 3'-O-t-butyldimethylsilylthymidine (0.34 mmol) and n-butyl-lithium (0.30 mmol in n-hexane) in 2 ml dry THF at -60°C, a solution of 11 (0.16 mmol in 2 ml THF) was added. After 4 h the solution was heated to 15°C and quenched with an excess of the pyridinium

form of Dowex 50W-X8. After separating the p-nitrophenolate precipitate and washing with pyridine, the organic layer was separated and washed with water. The solvent was evaporated and the residue was co-evaporated several times with toluene which was then redissolved in a small amount (5 ml) of chloroform:ethylacetate (1:1). This was then added (at -20°C) dropwise into n-pentane to afford the nucleotide thiophosphoroanilidate 12 which shows two diastereomeric ^{31}P signals at 57.10 and 57.30 ppm (53:47) diastereomeric ratio in C_6D_6).

Attempted Conversion of Thiophosphoranilidate 12 to Dithiophosphate 13. To a suspension of NaH (0.16 mmol) in dioxane and DMF (6 ml/1:1) was added, dropwise, at 25° C, a solution of the anilidate 12 (0.08 mmol) in dioxane (3 ml). Reaction was accompanied by the evolution of hydrogen and formation of a green precipitate. The reaction mixture was stirred at room temperature for the next hour and carbon disulfide (3 ml) was added dropwise. An additional 1 h of stirring at the same temperature was accompanied by a color change of the precipitate, containing crude dithiophosphate, 13, to a light brown. Upon cooling, the reaction mixture was treated with the pyridinium form of Dowex 50 X8 and filtered.

Efforts to purify 13 were unsuccessful. Thus a portion of the precipitate was suspended in 3 ml H_2O (pH 5) which was accompanied by a strong odor of H_2S. The suspension was stirred for 30 min (rt) and was filtered off and washed with water. The filtrate was evaporated and the residue was examined by ^{31}P NMR. The spectrum shows the diastereomer signals of a monothiophosphate species at 67.75, 67.50 ppm in D_2O and additional peaks at 3.10 and -91.75 ppm.

Some indication of partial conversion of the anilidate 12 to the dithiophosphate, 13 in the precipitate was suggested by trapping 13 as the methyl triester, 6. This was achieved by suspending the precipitate in benzene (3 ml) with addition of methyl iodide (molar excess). The suspension was stirred for 2 h (30°C), cooled and the precipitate was filtered and washed with benzene. The filtrate was evaporated and the residue was examined by ^{31}P NMR. The spectrum shows the diastereomer signals of the methyl ester (6, R=CH_3) of the dithiophosphate (δ = 94.5, 94.3 ppm) and two additional minor signals at 3.10 ppm and -91.75 ppm Further purification or characterization was not attempted.

NMR. The ^{31}P one-dimensional NMR experiments and the two-dimensional ^{31}P-^{1}H heteronuclear correlation experiments were acquired on a Varian XL-200 200 MHz spectrometer. The proton one-dimensional spectra, the two-dimensional pure absorption phase NOESY spectra, and the two-dimensional TOCSY spectra were acquired on a Varian VXR-600 600 MHz spectrometer. The proton spectra were referenced to H_2O at 4.76 ppm. The ^{31}P spectra were referenced to trimethylphosphate at 0.000 ppm.

The ^{1}H one-dimensional spectrum of the dithiophosphate decamer was acquired with a sweep width of 6300 Hz, 16K data points, and a relaxation delay of 3.0 s. The ^{31}P one-dimensional spectra of the dithiophosphate decamer were obtained with a sweep width of 15361 Hz, 16000 data points and a total recycle time of 1 s.

Two-dimensional Pure Absorption Phase[27] *NOESY Spectra* of the dithiophosphate decamer were acquired at mixing times 100, 200 and 300 ms. The 300 ms mixing time NOESY spectrum was collected for the assignment of the proton NMR and to measure the volumes of the proton-proton NOE crosspeaks in order to generate the NOE constraints for the structural studies. The NOESY spectra were acquired with a sweep width of 6300 Hz, 2048 points in the t2 dimension and 256 increments in the t1 dimension. Sixteen transients were collected for each t1 increment. The spectra were acquired with the sample non-spinning, a relaxation delay of 3 s, and no saturation of the water was used. The data were processed with 2K of zero-filling in the t2 and t1 dimension. A minimally shifted cosine-bell window and a Gaussian apodization function were used in both dimensions.

The TOCSY spectrum using a 60 ms spin locking time was acquired to assign the H5-H6 cytosine protons and the CH_3-H_6 thymidine protons ring. The spectrum was acquired with a sweep width of 6300 Hz, 2048 points in the t2 dimension and 256 increments in the t1 dimension. Sixteen transients were collected for each of the t1 increments. The spectrum was collected with the sample non-spinning, a relaxation delay of 3 s, and no saturation of the water was used. The spectra were processed with 2K of zero-filling in the t1 and in the t2 dimension A cosine-bell and a Gaussian apodization function were applied in both the t1 and t2 dimensions.

A $^{31}P/^{1}H$ Pure Absorption Phase Constant Time (PAC) version[28,29] of the Kessler-Griesinger Long-Range Heteronuclear Correlation (COLOC) experiment[30] was conducted on the dithiophosphate decamer. Two PAC spectra (^{31}P observed) were acquired The first one with a sweep width in the t2 dimension of 157.1 Hz centered around -4 2 ppm (for the phosphate ester region) and the second one with a sweep with of 383.5 Hz centered around 109.8 ppm (for the dithiophosphates). In both cases the sweep width in the the t1 dimension was set to 611.2 Hz in the H3′, H4′ H5′ region. The spectra were collected with 128 transients for each of the 64 FIDs with 256 data points of resolution. A 90° pulse of 8 μs for phosphorus and 80 μs for protons was used. The preacquisition delay was 2 s, the constant delay (CD) was 0.041s and the refocusing delay (RD) was 0.028 s. A first-order phase correction of 12,075° was used in the t2 dimension. The data was processed with 128 zero filling in the t1 dimension and 512 zero-filling in the t2 dimension. A line broadening of 2 was applied in both the t1 and the t2 dimension.

NOESY Distance Restrained Molecular Mechanics/Dynamics Calculations of the Dithiophosphate Decamer. The program MIDAS[31] operating on a Silicon Graphics Iris 3030 workstation was used for initial construction of the dithiophosphate decamer hairpin loop. The standard AMBER3 force-field parameters[32] were used for energy minimization of the model-built hairpin loop decamer. In later restrained MD calculations, the force field parameters for a dithiophosphate were incorporated into the AMBER3 force field. These were based upon GAUSSIAN86[33] calculations of a model dimethyl thionophosphate at the 3/21G basis set level (D. Gorenstein, unpublished). NOESY distance constraints were incorporated into the potential energy force field through addition of a flatwell potential [34,35] The unconstrained energy refined, model-built structure with 99 NOESY distance constraints for the single strand loop from the 300 ms NOESY spectrum was then energy refined until a rms

gradient of 0.1 kcal/mol-Å was achieved or until the change in energy was less than 1.0×10^{-7} kcal/mol for successive steps. The energy minimization and dynamics used the flatwell distance constraints potential with an initial force constant of 5 kcal/mol with a permitted distance error of ±15%. A residue based cut-off and a distance dependent dielectric function were used. The latter approximates a solution dielectric constant for a gas phase minimization. An 8.5 Å distance cut-off was used for non-bonded pairs interactions. The 1-4 van der Waals and the 1-4 electrostatic interactions had a scale factor of 2.0. A full conjugate gradient minimization was calculated with an initial step length of 5×10^{-4} and a maximum step length of 1.0. The shake routine was not used. Refinement utilized separate 6 ps cycles of AMBER3 molecular dynamics as previously described.[34,35]

Hybrid Matrix/MORASS Refinement of Structures

A relaxation matrix program (*MORASS*· *M*ultiple *O*verhauser *R*elaxation *A*nalysi*S* and *S*imulation)[36,37]; the program is available upon request) was used to calculate volume and rate matrices as well as implement the hybrid matrix methodology. The well-resolved and measurable crosspeaks in the NOESY spectrum replace the corresponding crosspeaks in the calculated volume matrix, while overlapping or weak crosspeaks and diagonals are from the calculated spectrum. This hybrid volume matrix, V^{hyb}, is then used to evaluate the rate matrix, whose off-diagonal elements include the effects of spin diffusion. Distances derived from this hybrid relaxation rate matrix (we assume a single isotropic correlation time of 3.0 ns) are then utilized as distance constraints in a 6 ps restrained molecular dynamics simulation. Energy minimization of the averaged, last 1 ps structures derived from molecular dynamics completes one cycle of refinement. This process is repeated until a satisfactory agreement between the calculated and observed crosspeak volumes is obtained. As shown by our laboratory[35,38] and Kaptein and coworkers[39,40], 3 or more iterations appear to be adequate to achieve convergence to a "refined" structure

Convergence is monitored using eqn. 1.

$$\%\mathrm{RMS}_{vol} = \sqrt{\frac{1}{N}\sum_{ij}\left(\frac{v_{ij}^{a} - v_{ij}^{b}}{v_{ij}^{a}}\right)^{2}} \cdot 100\% \qquad (1)$$

where % RMS_{vol} = % RMS_{the} when a = calculated the volume and b = the experimental volume, % RMS_{vol} = % RMS_{exp} when a = the experimental volume and b = the calculated volume.

Results and Discussion

Comparison of Various Synthetic Routes to Dithiophosphates

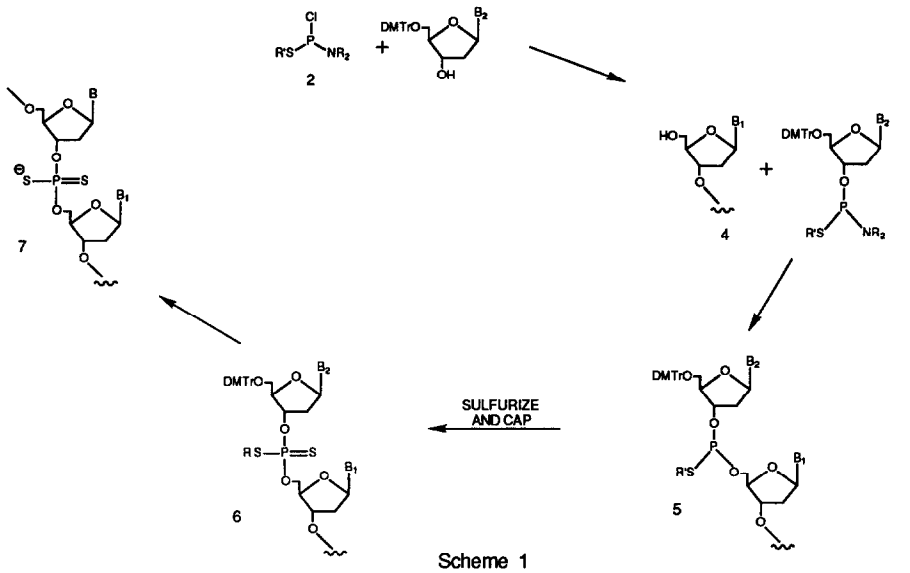
Recent interest in the application of dithiophosphate oligonucleotides as antisense analogues for anti-viral application has resulted in the development of thiophosphite triester[7–14], dithiophosphoric acid[19] and H-phosphonothioate[15–18] synthetic schemes. We have explored both the P(III) and P(V) synthetic routes shown in Schemes I and II

Thiophosphoranilidate Route The design of the P(V) phosphoramidate methodology in synthesizing dithiophosphate oligonucleotides is based upon a similar strategy for the synthesis of monothiophosphate analogues described in ref. 41. While the thiophosphoranilidates, 11 and 12 may be synthesized in good yield and purity, all efforts to prepare pure dithiophosphates by this route have been unsuccessful. However we have been able to trap the dithiophosphate by alkylation with methyl iodide to demonstrate the partial conversion of the thiophosphoanilidate 12 to the desired crude dithiophosphate 7.

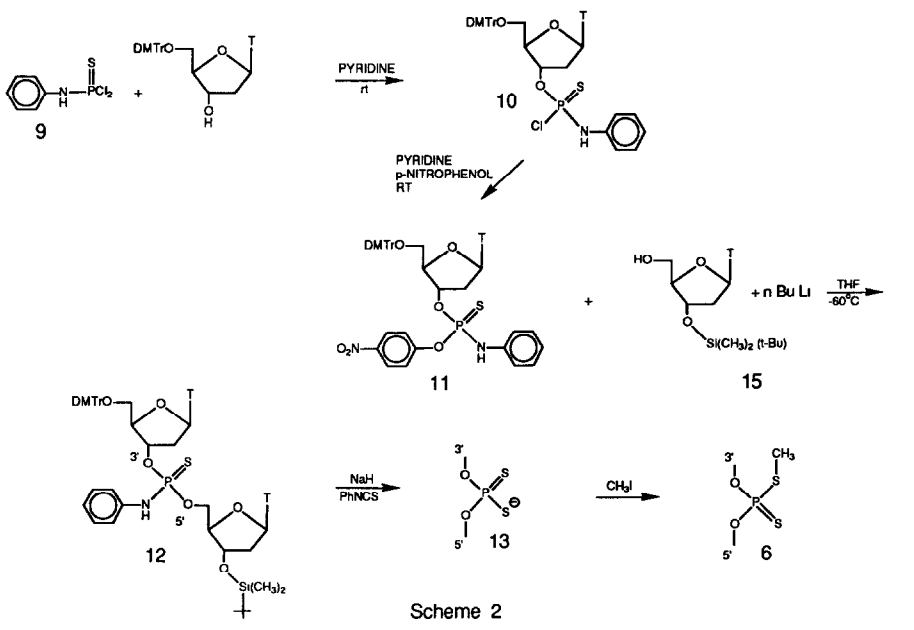
Thioalkylphosphoramidite Route Several routes to the synthesis of various thiophosphoramidite intermediates for preparation of dithiophosphate oligonucleotides have been demonstrated.[7–9,12] Our laboratory[9] has shown that chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, can be readily converted into deoxynucleoside thioalkylphosphoramidites such as 4 (Scheme I).

Several strategies have been investigated for the synthesis of monochloro-N,N-diisopropylaminomercaptophosphine, 2. Dichloro-N,N-diisopropylaminophosphine, 1, in contrast to alkyloxydichlorophosphines, is mildly reactive towards thiols. Several procedures and catalysts were explored to develop optimal reaction conditions for the introduction of the mercapto group into the phosphoramidites. The best yield of the intermediate 2 was obtained by the reaction of dichloro-N,N-diisopropylaminophosphine, 1, with sodium thiomethoxide (1 equiv) in the presence of two catalysts – aluminum trichloride and potassium iodide

The crude chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, can be stored at −18 °C under an inert, dry atmosphere for at least several months without any decomposition. In contrast, the N,N-diisopropylaminodithio-



Scheme 1



Scheme 2

methoxyphosphine, 3, undergoes a Michael-Arbuzov reaction to the extent of roughly 50% after 6 weeks at −18 °C. However, a serious problem of all monofunctional phosphitylating agent such as chloro-N,N-dialkylaminomethoxyphosphine [20,42,43] and chloro-N,N-diisopropylaminothiomethoxyphosphine, 2, is their sensitivity towards hydrolysis and air oxidation which requires careful handling.

Caruthers and coworkers[7,8] have shown that the phosphorothioamidite intermediate 4 could be prepared in a simple one-pot synthesis via reaction of suitably protected deoxynucleoside with S-(alkyl)-N,N,N',N'-(tetramethyl)-phosphorothiodiamidite. Because of sensitivity of this reaction to traces of oxygen and water, we have found that better purity can be obtained by running these reactions in an inert atmosphere dry box.

Preparation of Dithiophosphates from Thiophosphoramidites The thiophosphoramidite reagents, 4, may be readily coupled with 3'-protected nucleosides in solution or on glass supports. As shown by our laboratory[9] and Caruthers[7,8] and coworkers 1H-tetrazole appears to be the most effective catalyst for this coupling. In solution the reaction may be readily monitored by ^{31}P NMR spectroscopy. Thus addition of excess 3'-O-(t-butyldimethylsilyl)thymidine (or acetyl) protected thymidine and 1H-tetrazole at r.t. resulted in the complete disappearance over several hours of the two ^{31}P signals at 164.9 and 163 1 ppm, which are replaced by two new signals at 190.7 and 191.3 ppm (55:45 ratio). The later are assigned to a R_P and S_P diasteromeric mixture of O-(3'-O-t-butyldimethylsilylthymidin-5'-yl O-(5'-dimethoxytritylthymidin-3'-yl) S-methyl thiophosphite, 5. The best yield of the thiophosphite was obtained by reaction for 60 min.

After sulfurization with a large excess of sulfur in pyridine the 3',5'-dithymidine methyl phosphorodithioate diastereomers, 6 were observed as two ^{31}P signals at 95.7 and 95.4 ppm (benzene-d_6). The acetyl protected dithioate triester ^{31}P signals appear at 96.8 and 97 2 ppm (benzene-d_6). The sulfurization reaction was rapid, and after 10 min. at ambient temperature ca. 5% of the unreacted dinucleoside thiophosphite was still present.

S-demethylation of 3',5'-dithymidine methyl phosphorodithioate was accomplished by reaction with thiophenol/diisopropylethylamine for 40 hours at rt to yield the 3',5'-dithymidine phosphorodithioate (^{31}P, 114.9 ppm), 7.

Solid Phase Synthesis of Dithiophosphate Modified Oligonucleotides

We have also prepared various dithiophosphate oligonucleotide analogues of d(CGCTTAAGCG) possessing various dithiophosphate linkages in one or more sites by a manual solid phase methodology similar to that described in ref. 7, 23, 44. Several dithiophosphoryl analogues for the decamer, d(CGCTpS_2TpS_2AAGCG), d(CGCTpS_2TpS_2ApS_2ApS_2GCG) and d(CpS_2GpS_2CpS_2TpS_2TpS_2ApS_2ApS_2GpS_2CpS_2G) have been prepared in ca. 80% yield before purification.

^{31}P NMR Spectra of dithiophosphate oligonucleotides

The large downfield shift (ca. 110 ppm) of the dithiophosphate groups relative to the phosphate moiety (ca. -4 ppm) provides a convenient monitor of the success of the dithiophosphate analogue synthesis. Air oxidation rather than sulfur oxidation of the thiophosphite, 5 is readily identified by the ^{31}P signals from monothiophosphate species at ca. 50-60 ppm. As shown in Figure 1, multiple dithiophosphoryl substitutions into an oligonucleotide show the expected reduction in the number of phosphoryl-oligonucleotide signals with replacement by new dithiophosphoryl signals.

The assignment of the resonances in the ^{31}P spectrum of the TT-pS_2-decamer (Figure 1A) is based upon a *P*ure *A*bsorption phase, *C*onstant time $^1H/^{31}P$ heteronuclear correlated spectrum, PAC[28] (Figure 1B) The PAC experiment contains crosspeaks between the phosphorus resonance and the H3', H4' and H5'/H5'' protons via long-range coupling. Thus, with the known H3' chemical shift assignments from the 2D-NOESY spectrum (see

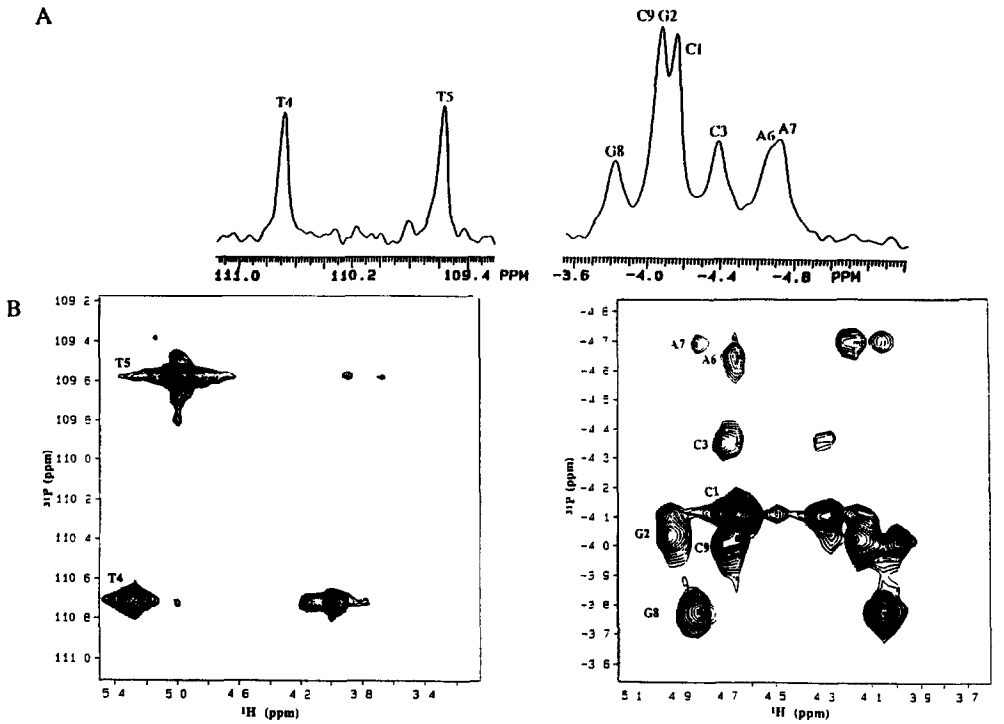


Figure 1: A. ^{31}P NMR spectra and phosphate assignments of TT-pS_2-decamer. (Numbering corresponds to phosphate position from the 5′-end of the duplex.) B. Two-dimensional ^{31}P-^{1}H PAC heteronuclear correlation NMR spectrum of duplex TT-pS_2-decamer at 200 MHz (^{1}H)

below) the corresponding phosphorus resonances were assigned (Figure 1A) The TT-dithiophosphoryl decamer phosphorus assignments are listed in Table I.

^{1}H NMR Assignments of the Dithiophosphate Decamer in Low Salt Buffer

The 1D and 2D proton NMR spectra of the dithiophosphate decamer analogue (5 mM single strand) in 50 mM KCl, 10 mM phosphate, pH 6.8 and 5.2, and ambient temperature are quite different from that of the parent decamer [45]

The proton spectrum of the dithiophosphate decamer analogue in this low salt buffer was assigned through analysis of two-dimensional TOCSY and NOESY spectra (Figure 2) following the sequential assignment methodology.[24,4] The TOCSY spectrum provided the identification of the H5 and H6 protons of the three cytosines Once the cytosines were located their respective H1′ protons were easily identified in the NOESY spectrum by comparison with the chemical shifts obtained from the TOCSY spectrum (spectra not shown) The TOCSY spectrum also provided the unambiguous identification of the two thymines through coupling of T(H6) to its methyl group (1.88 and 1.68 ppm).

The 300 ms NOESY spectrum of the base to H1′ region illustrates the sequential assignments of each base proton (Figure 2A). The base to H1′ connectivity along the entire backbone from C1 to G10 is clearly observed in the NOESY connectivity with interruption only for the T4 H1′ – T5 H6 cross peak These assignments were confirmed in the base to H2′/H2″ region. This region of the spectrum shows connectivities between the H8 or

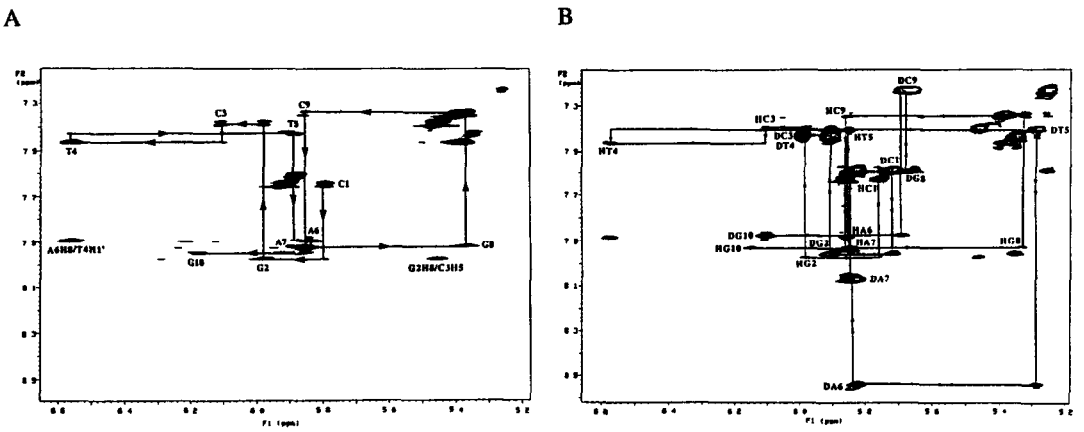


Figure 2: A. Pure absorption phase 300 ms mixing time, $^1H/^1H$ NOESY NMR spectrum of duplex TT-pS_2-decamer dithiophosphate decamer at 600 MHz, 50 mM KCl, 10 mM phosphate, pH 6.8, showing the base H8/H6 and deoxyribose H1′ region. B. Base to H1′ expanded region NOESY spectrum in 210 mM KCl, 50 mM Pi, pH 6.8. This sample consists of a mixture (1:2) of both hairpin (H) and duplex (D) forms of the TT-pS_2-decamer. The sequential connectivity of the base H8/H6 and deoxyribose H1′ is diagrammed.

H6 base proton and the H2′/H2″ protons of its own deoxyribose rings as well as those of the the next residue towards the 5′ side. T5 H6 shows only cross peaks to its own methyl group and H2′/H2″ protons and very weak (or absent) cross peaks to protons on neighboring nucleosides. Another interesting point in this spectrum is the unusual chemical shifts of T5 H2″ at 1.16 ppm and the methyl group of T5 at 1.88 ppm.

Another region of interest that confirms these assignments was the base to H3′ region that shows clear connectivities from C1 to T5 and from A6 to G10. The interuption of the connectivity between T5 and A6 is of particular interest At this point the connectivity is simply reversed, instead of there being a connectivity from T5 H6 to A6 H3′, there is a connectivity form A6 H8 to T5 H3′ indicating clearly that the A6 base is in close contact with the sugar ring of T5 and that the T5 base is far from the sugar ring of A6. Further assignment of the spectrum through the sequential methodology was straightforward. The nearly complete assignment of the dithiophosphate

Table I: d(CGCTpS_2-TpS_2-AAGCG) $^{31}P^a$ and $^1H^b$ Chemical Shifts (ppm)^c

Atom	P-31	H1'	H2'/H2"	H3'	H4'	H5'/H5"	H5	H6/H8
Cyt 1	-4.14	5.78	2.38/1.94	4.67	4.05	3.07/3.70	5.90	7.67
Gua 2	-4.07	5.95	2.74/2.68	4.94	4.33	4.05/3.95	-	7.95
Cyt 3	-4.39	6.05	2.52/2.16	4.72	4.22	4.12/4.12	5.41	7.40
Thy 4	110.67	6.40	2.74/2.40	5.33	4.35	4.22/4.15	-	7.47
Thy 5	109.47	5.96	1.80/1.16	5.03	4.23	4.02/4.02	-	7.59
Ade 6	-4.65	5.76	2.33/2.22	4.69	3.96	3.55/3.55	-	8.00
Ade 7	-4.72	5.96	2.78/2.68	4.84	4.33	4.00/4.12	-	7.98
Gua 8	-3.82	5.44	2.58/2.50	4.86	4.22	4.07/4.07	-	7.90
Cyt 9	-4.07	5.89	2.34/1.88	4.73	4.13	4.03/4.03	5.34	7.37
Gua10	-	6.15	2.64/2.33	4.64	4.16	4.03/4.03	-	7.92

a P-31 chemical shifts referenced to trimethyl phosphate at 0.000 ppm.
b H-1 chemical shifts referenced to HDO at 4.76 ppm.
c pH=5.4

decamer's ^{1}H NMR spectrum is listed in Table I.

Comparison of the base to H1′ region of the NOESY spectrum to the same region of the parent oligomer shows that in the modified oligomer one H1′ is significantly more upfield (G8 H1′, 5.44 ppm instead of 5.94 ppm) and another H1′ is downfield (T4H1′, 6.40 ppm instead of 6.04 ppm). The order of the bases proton is almost unchanged with the AH8 and GH8 protons being most downfield and the CH6 and TH6 being upfield. However T5 H5 and G8 H8 appear at quite different chemical shifts.

A striking feature of the base to H1′ NOESY spectrum (Figure 2A) is the observed connectivity between A6 H8 and T4 H1′ (dashed line). This type of connectivity does not exist in regular A or B DNA where the distance between bases i and i+2 is too large This connectivity clearly indicates an unusual structure that would place A6 H8 in close proximity to T4 H1′ Recall also that T5 H5 had very few NOESY connectivities to its neighbors. These observations are consistent with a structure in which the base of T5 loops out in a possible single strand hairpin loop or bulged duplex

In addition the T5H5 proton appears much broader suggesting chemical exchange of the T5 base. Other resonances show significant line broadening as well. Among the resolved peaks, T4H1′, T4H3′, T5H1′, T5H3′, A6H1′, A7H1′, G8H1′ are broader than the other resonances suggesting that increased conformational flexibility exists in this loop region. The NOESY crosspeaks suggest that the T5 base stacks out of the structure, whereas the A6 pair stacks inside The main uncertainty concerns the A7 base which could either stack in or loop out based upon a qualitative analysis of the NOESY spectrum. However the cross-peaks form A7H8 to G8H1′ and from A7H8 to A6H2′ and A6H2″ generally support a structure with A7 stacking in A NOESY spectrum in water shows only two peaks at ca. 13 ppm (ratio of 2 1), suggesting that only C-G base pairs are present. Thus there is no evidence for A-T base-pairs, in support of the hairpin loop conformation.

NMR Structural Refinement of the Thiophosphoryl Decamer from 2D NOESY Distances

Evaluation of interproton distances from a 2D-NMR NOESY spectrum has generally relied on the so-called "two-spin approximation".[50,51] The approximation requires that the NOESY derived distances be obtained from vanishingly short experimental mixing times where the rate of build-up of the NOE crosspeak intensity is ca. linear and the effects of spin diffusion are minimal. Because most of the structurally important longer range NOEs are not observed at these short mixing times, the use of the two-spin approximation has raised concern over the validity of refined NMR structures derived by this methodology.[35,38,52] The effects of spin diffusion increase with an increase in mixing times and at a mixing time of 300 ms can introduce significant errors in measured distances.[35,37,52] Therefore, a hybrid relaxation matrix procedure was employed to correct for multi-spin effects at this longer mixing time. Refinement of the structure using restrained molecular dynamics and a relaxation matrix program (*MORASS*: *M*ultiple *O*verhauser *R*elaxation *A*nalysis*S* and *S*imulation[36,37]) to calculate volume and rate matrices as well as implement the hybrid matrix methodology as previously described.[38]

The distances derived from the NOESY spectrum were used to model a structure for the decamer. The AMBER3[32] molecular mechanics/dynamics program was used for energy minimization of a model-built hairpin loop decamer. A total of 99 NOESY distance constraints were then incorporated into the AMBER potential energy force field through addition of a flatwell potential[34,35] and the structure reminimized. Only those crosspeaks that could be adequately resolved from overlapping peaks were included

Imino hydrogen bond constraints were added. The typical refinement follows the iterative hybrid matrix/MORASS/restrained molecular dynamics methodology incorporating the NOESY distance constraints. Initial structures were used to calculate a theoretical NOESY volume matrix which was merged with the experimental NOESY matrix using MORASS. After the volume matrix is calculated, the data sets are scaled by using the

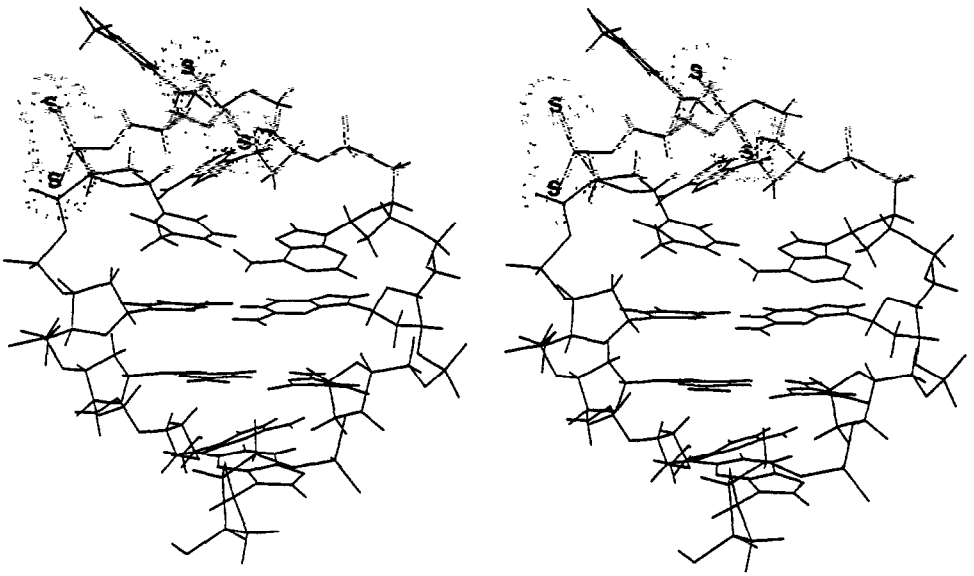


Figure 3: Stereoview of the final NOESY-distance restrained, molecular dynamics structure obtained through the hybrid matrix/MORASS/MD refinement of the TT-pS_2-decamer model built hairpin loop. The van der Waals surface of the sulfurs is shown as is the NOESY connectivity between the A H8 and T4 H1′ protons (dashed line)

resolved cytosine H5-H6 NOEs and H2′-H2″ fixed distance crosspeaks The distance constraining pseudo-force constants were gradually increased from 5 to 30 kcal/mol/Å^2 and the estimated distance error brackets were gradually decreased from ±15 to ±5.0%. The TT-pS_2-decamer structure was refined from a model built structure although the distance geometry program DISGEO was shown to generate comparable loop structures based upon these constraints (M. Piotto, unpublished)

Figure 3 shows the structures after the 18th, 6 ps, merge matrix iteration cycle (total 114 ps MD) starting from the initial hairpin model. Figure 3 is a stereoview of the final refined structure from the MORASS/AMBER protocol.

The convergence in the MORASS refinement was monitored by the %RMS_{vol}. At this preliminary stage of refinement the %RMS_{the} has decreased from 282 to 50%. This is similar to the quality of MORASS refinement of other oligonucleotides.[35,38,53,54]

Comparison with the Parent Decamer

Thus, in contrast to the parent palindromic decamer sequence[45] which has been shown to exist entirely in the duplex B-DNA conformation under comparable conditions (100 mM KCl, 10 mM strand concentration), the dithiophosphate analogue forms a hairpin, even at twice the strand concentration. Polyacrylamide gel electrophoresis shows that the dithiophosphate analogue runs faster than the parent duplex, also consistent with the hairpin loop for the former However, at higher salt concentrations (200 mM), four ^{31}P signals for the dithiophosphoryl groups of the dithiophosphate analogue are observed The additional pair of ^{31}P dithiophosphate signals suggests that the dithiophosphate analogue forms a 1:2 mixture of duplex and hairpin loop. This has been confirmed by analysis of the ^{1}H 2D NMR spectra of the mixture, which clearly shows the expected connectivity and chemical shifts for both duplex and hairpin (Figure 2B; M. Piotto and J. Granger, unpublished)

The destabilization of the duplex form for the dithiophosphate is possibly attributable to unfavorable dithiophosphate electrostatic repulsion in the duplex form. In the hairpin the thymidine dithiophosphates do not interact with an adjacent phosphate group across the major and minor grooves. The P-S bond length is ca. 0.5 Å longer than the phosphoryl P-O bond length (based upon ab initio calculations with a 3/21 G basis set; D. G. Gorenstein, unpublished) and the larger van der Waals radius for sulfur are presumably responsible for the increased electrostatic destabilization of the duplex form. As shown in Figure 3, the hairpin structure also separates the large dithiophosphates (van der Waals surface for the sulfurs are indicated) along the strand to a much greater degree than is possible in the duplex form

These results demonstrate the importance of electrostatic interactions in the relative stabilization of duplex and hairpin DNA. It also raises potential implications for design of monothiophosphate and dithiophosphate antisense analogues.

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