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18. Some residues are so big that they are greater than half the cut-off distance. In this case, *Discover* stops that process. So larger cut-off distance should be adopted. However, computing time will increase in third power as cutoff distance increases linearly. Besides, even cutoff 20 Å made a trouble during the computation, though it had worked well at the start of process. As size of amino acid rather varies during minimization, cutoff value should be sufficient during whole process. The option of IGRPCK=0 makes it possible to continue the process with ignoring the instant expand of residue, if the process did not have any trouble at the start of MM.
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24. Of course this needs to be explained more. The equilibrium constant, K in eq. (3) is unitless but inhibition constant K_i has unit of nM. Therefore $RT\ln(K_i/K_i')$, K_i' some calibration constant, has closer relation to $E_{binding}$. So the comparison between $\delta(RT\ln K_i)$ and $E_{binding}$ is more desirable to that between $RT\ln K_i$ and $E_{binding}$.

Synthesis and Configuration Analysis of Diastereomers of 5′-O-(2′-Deoxycytidyl)-3′-O-Thymidyl Phosphorothioate

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A procedure is described for the synthesis of the title compound *via* phosphotriester intermediates. The preparation of R_p and S_p diastereomeric dinucleotide of d[Cp(S)T] was performed by the condensation of the protected deoxycytidine, the protected thymidine, 2,5-dichlorophenylphosphorodichloridothioate and 1-hydroxybenzotriazole in THF. Their designation of configuration at phosphorus as R_p and S_p follows from anaylsis of ^{31}P NMR spectroscopy and reverse-phase HPLC and the stereospecificity in the hydrolysis catalyzed by Nuclease S1 and snake venom phosphodiesterase. Diastereomerically pure R_p and S_p d[Cp(S)T] were utilized to synthesize oligonucleotides containing the XhoI recognition sequence with a phosphorothioate group at the cleavage site.

Introduction

Diastereomeric phosphorothioate analogues of nucleotide are important tools for elucidation of the stereochemistry of action of different classes of enzymes. For instance, the stereochemical course of action of RNase A.[1,2] To obtain a more complete insight into enzyme-substrate interactions of exo- and endonucleases in general, one needs the two diastereomeric phosphorothioate analogues of an appropriate dinucleoside monophosphate in their optically pure form.

To study the stereochemical course of reaction catalyzed by XhoI restriction endonuclease, we need to synthesize a oligodeoxynucleotide which contains the XhoI recognition sequence with a phosphorothioate group at the cleavage site. Deoxyoligonucleotides which contain phosphorothioate linkage at cleavage site of restriction endonucleases can be used to elucidate the stereochemical course of reaction catalyzed by restriction endonucleases. For instance, the sterochemical courses of action of EcoRI[3,4] and EocRV[5] have been established using oligonucleotide containing the appropriate recognition sequence with phosphorothioate internucleotidic linkage of known absolute configuration. Thus we have synthesized opically pure diastereomers of 5′-O-(2′-deoxycytidyl)-3′-O-thymidyl phosphorothioate (d[Cp(s)T]), which can be used as a dimeric building block to make oligonucleotides containing the appropriate recognition sequence of XhoI restriction endonuclease with phosphorothiate internucleotidic linkage of cleavage site.

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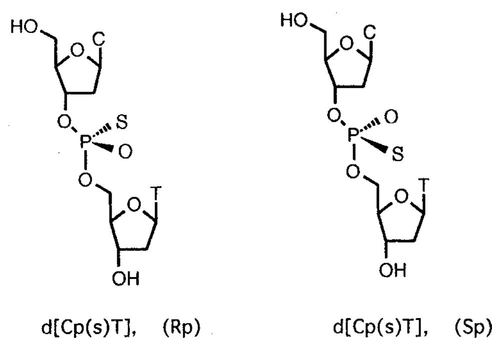
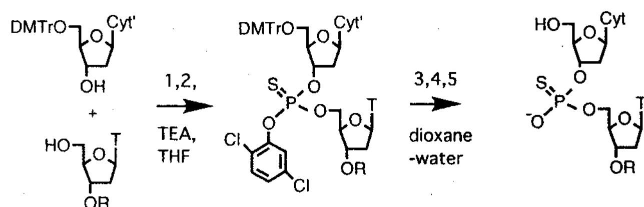


Figure 1. Configuration of the S_p and R_p diastereomers of the dinucleoside phosphorothioate, d[$C_{p(S)}$T].



(1) 2,5-dichlorophenylphosphorodichloridothioate, (2) 1-hydroxybenzotriazole, (3) phenyldihydrogenphosphate, (4) syn-2-nitrobenzaldoxime, (5) tetramethylguanidine, Cyt'; 4-N-benzoylcytosine, DMTr; 4,4'-dimethoxytrityl, R; p-chlorophenoxyacetyl

Scheme 1. Synthesis of Protected Diastereomeric Dinucleoside Phosphorothioates.

In this paper we described the synthesis, separarion, configurational analysis of diastereomeric dinucleotide phosphorothioate, d[Cp(s)T] (Figure 1) and utilization of the optically pure diastereomers for synthesis of oligonucleotides containing the XhoI recognition sequence with a phosphorothioate group at the cleavage site.

Results and Discussions

The desired phosphorylating agent, 2,5-dichlorophenylphosphorodichloridothioate was prepared following modifications of the reported procedure[6] in 72% yield. Both diastereomers of phosphorothioate containing d[Cp(s)A] dimer were prepared by a modification of Kemal's procedure[7] which uses phosphotriester approach leads to high yield. The d[Cp(s)T] dimer was prepared by condensing by 4-N-benzoyl-5′-dimethoxytrityl-2′-deoxycytidine and 3′-O-[(p-chlorophenoxy) acetyl]thymidine using 2,5-dichlorophenyl-phosphorodichloridothioate and 1-hydroxybenzotriazole as condensing agent in THF (Scheme 1). Purification and separation of diastereomers were simultaneously achieved by short column chromatography over silica gel eluting with ethanol in chloroform. Contrast to separation of diastereomers of 5′-O-(2′-deoxyadenosyl)-3′-O-(2′-deoxyadenosyl) phospho-rothioate (d[$A_{p(S)}$A]),[8] column chromatography must be performed without removal of the (p-chlorophenoxy)acetyl protecting group. When we separated diastereomers of (d[$A_{p(S)}$A]), we had to remove the (p-chlorophenoxy)acetyl protecting group before chromatography. But attempt to separate diastereomers of d[$C_{p(S)}$T] without the protecting group was not succeeded. After chromatography, the diastereomers were checked with high performance thin layer chromatography (HPTLC) plates because the difference in R_f values of two diastereomers were too small to be separated on regular TLC plates. Short column chromatography afforded the pure higher R_f [0.36, $CHCl_3$-EtOH(95 : 5 v/v)] diastereomer in 25.1% yield and the pure lower R_f (0.31) diastereomer in 10.6% isolated yield. ^{31}P NMR spectroscopy of the diastereomers at 25 ℃ in $CDCl_3$ showed resonance at 63.12 and 62.13 ppm, respectively (Figure 2). After removal of the (p-chlorophenoxy)acetyl protecting group by brief treatment with ammonia, the diastereomers were treated first with N^1, N^1, N^3, N^3-tetramethylguanidine in dioxane-water at room temperature to unblock the internucleotide linkage and then aqueous ammonia to remove benzoyl group. ^{31}P NMR spectroscopy of the resulting fully unblocked d[Cp(s)T] showed resonance at 56.42 and 55.92 ppm, respectively. Since it is known that the S_p diastereomer of dinucleotide phosphorothioates resonates at higher field than the R_p diastereomer,[8~10] this established that the higher R_f was a diastereomer with the S_p configuration. Confirmation of this results comes from reversed-phase HPLC analysis of the unblocked mixture in which the higher R_f diastereomer elutes before the lower one (Figure 3). Again the R_p diastereomer of dinucleotide phosphorothioates is known to elute before the S_p in reversed-phase HPLC system.[8~10] The above result were confirmed by studying the hydrolysis of these diastereomers catalyzed by nuclease P1, which is

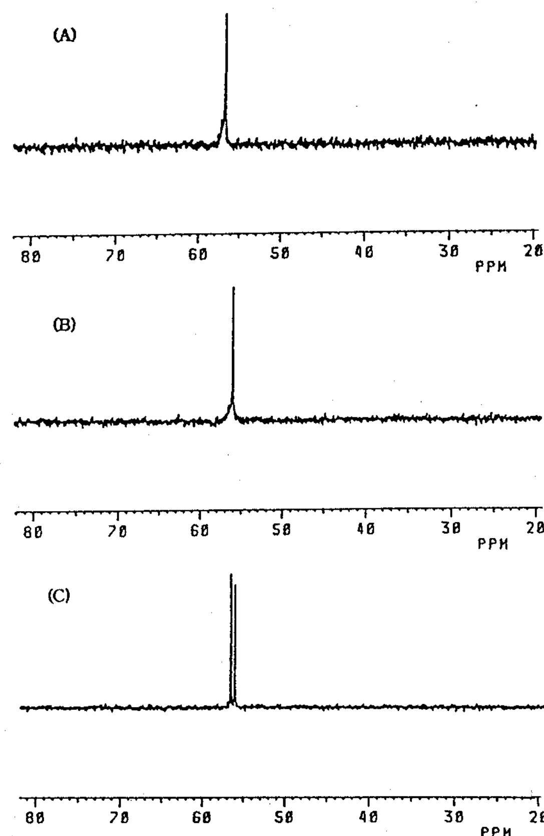


Figure 2. ^{31}P NMR spectra of the diastereomers of d[$C_{p(S)}$T]: (A) (R_p)-d[$C_{p(S)}$T] (55.92 ppm), (B) (S_p)-d[$C_{p(S)}$T] (56.42 ppm), (C) a mixture of (Rp)- and (S_p)-d[$C_{p(S)}$T].

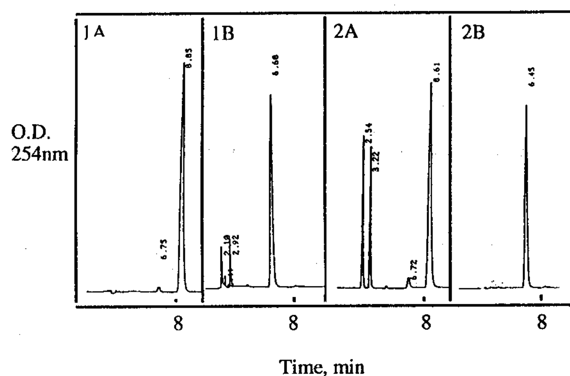


Figure 3. HPLC analysis of a partial enzyme digestion of d[$C_{p(S)}$T] (1A) lower R_f diastereomer with phosphodiesterase, (1B) higher R_f diastereomer with phosphodiesterase, (2A) lower R_f diastereomer with Nuclease S1, (2B) higher R_f diastereomer with Nuclease S1.

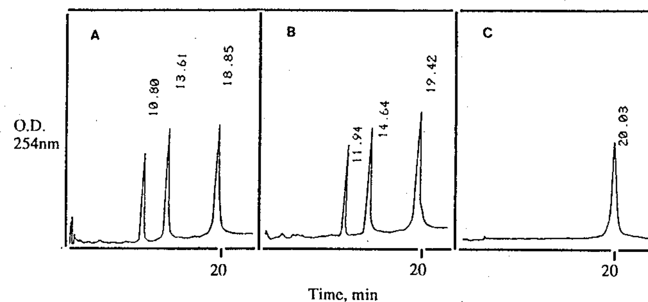


Figure 4. HPLC analysis of a partial XhoI digestion of oligonucleotides containing XhoI recognition sequence. (A) GATCTCGAGATC, (B) (R_p)-GATC$_{p(S)}$TCGAGATC, and (C) (S_p)-GATC$_{p(S)}$TCGAGATC. In all cases undigested oligomers elute latest.

known to hydrolyzes dinucleoside phosphorothioate of the S_p but not R_p configuration[11] and phosphodiesterase I, which hydrolyzes R_p, but not S_p configuration of diastereomer. The results presented in Table 1 clearly demonstrate that the fast-moving isomer (higher R_f) of triester corresponds to the dinucleoside phosphorothioate of the R_p configuration (Figure 1). The protected optically pure (R_p) and (S_p) diastereomers were used to synthesize dodecamers d[GATC$_{p(s)}$TCGAGATC] which contains the recognition sequence for Xho I with a phosphorothioate group at the cleavage site. HPLC analysis show the two diastereomers of d[GATC$_{p(s)}$TCGAGATC] synthesized from optically pure d[$C_{p(S)}$T] were pure without contamination by the other isomer, respectively. These both diastereomers elute later than normal oligonucleotide, GATCTCGAGATC on reversed-phase HPLC. The R_p and S_p configuration of d[Cp(S)T] were again confirmed by incorporating the dinucleoside phosphorothioates into oligonucleotides containing the XhoI recognition sequence with a phosphorothioate group at the cleavage site and analyzing the hydrolysis of the oligomers with restriction endonuclease XhoI. It is known that restriction endonucleases can hydrolyze the R_p diastereomer of the oligonucleotide which contains a chiral phosphorothioate group of the R_p configuration at cleavage site.[4,5] First, we tested oligonucleotide, GATCTCGAGATC which contains the XhoI recognition sequence without a phosphorothioate group at the cleavage site. It was hydrolyzed by XhoI ($t_{1/2}=\sim$10h). R_p-diastereomer of d[GATC$_{p(s)}$TCGAGATC] which was synthesized with R_p-d[Cp(s)T] was hydrolyzed by XhoI, but the rate of hydrolysis was slower than with normal oligonucleotide ($t_{1/2}=\sim$24h). S_p-diastereomer which was prepared with S_p-d[Cp(s)T] was not hydrolyzed by XhoI (Figure 4). The same results have been seen with EcoRI endonuclease[4] and EcoRV endonuclease.[5] The details of synthesis and characterization of two diastereomers of d[GATC$_{p(s)}$TCGAGATC] and the stereochemical course of the restriction endonuclease XhoI catalyzed reaction will be reported separately.

Experimentals

Nucleosides were obtained from Sigma Chemical Company. Pyridine, triethylamine and tetrahydrofuran were distilled over calcium hydride. Dioxane was distilled over Na. 2,5-Dichlorophenol, PCl_3 and $PSCl_3$ were purchased from Fluka Chemical Co. (Switzerland). Nuclease S1 was purcha-

Table 1. Analytical Data on Protected and Unprotected Dinucleoside Phosphorothioate

	^{31}P NMR (ppm)		R_T (min)	Nuclease P1 digestion	Phosphodiesterase I digestion	Configuration
protected *d*[Cp(s)T]	63.12^b	0.36	–	–	–	Rp
protected *d*[Cp(s)T]	62.13	0.31	–	–	–	Sp
protected *d*[Ap(s)A]^f	63.07	0.35	–	–	–	Rp
protected *d*[Ap(s)A]^f	62.76	0.34	–	–	–	Sp
d[Cp(s)T]	56.42^c		6.51^d	not cleaved	cleaved	Rp
d[Cp(s)T]	55.92^c		8.32^d	cleaved	not cleaved	Sp
d[Ap(s)A]^f	56.24		14.62^e	not cleaved		Rp
d[Ap(s)A]^f	55.68		16.40^e	cleaved		Sp
d[Gp(s)C]^g	59.59		11.2	not cleaved		Rp
d[Gp(s)C]^g	55.13		12.7	cleaved		Sp

^{a}HPTLC (5% Methanol in $CHCl_3$). ^{b}Measured in $CDCl_3$. ^{c}Measured in H_2O. dHPLC retention time in gradient I. ^{e}HPLC retention time in gradient II. ^{f}Data taken from reference 8. ^{g}Data taken from reference 12.

sed from KOSCO and sanke venom phosphodiesterase from Sigma Chemical Company. Restriction endonuclease XhoI was purchased from Promega. Thin layer chromatography was performed on Merck Kiesel $60F_{254}$ plates and HPTLC plates that were eluted with 5% methanol in chloroform. Merck Kieselgel 60H was used for column chromatography. The ^1H NMR and ^{31}P NMR spectra were measured with a Bruker 300-MHz spectrometer. An applied Biosystem HPLC system equipped with an UV-visible detector (model 783A) and with gradient pump system (model 400) was employed. In all cases, the reverse phase Jones ODS column (5 mm particle size, 250×4.6 mm) was used. Two buffer systems were used. To purify completely deblocked diastereomers, a linear gradient (flow rate 1.5 mL/min) consisting of 0.1 M triethylammonium acetate (TEAA), pH 7.0(A) and 0.1 M TEAA, pH 7.0, containing 60% CH_3CN(B) was used (t=0 min, 14% B; t=20 min, 40% B) (gradient I). To resolve nuclease S1 and snake venom phosphodiesterase digestion products of the diastereomers, linear gradient (flow rate 1.5 mL/min) prepared from 50 mM KH_2PO_4, pH 6.0(A) and 50 mM KH_2PO_4, pH 6.0, containing 30% CH_3CN(B) was used (t=0 min, 0% B; t=15 min, 50% B) (gradient II).

Chemical Synthesis

2,5-Dichlorophenylphosphorodichloridothioate. Following the procedure,[6] the desired phosphorylating agent, 2,5-dichlorophenylphosphorodichloridothioate was prepared by heating 2,5-dichlorophenylphosphorodichloridite, thiophosphoryl chloride, and sulfur in the presence of activated charcoal in 72% yield: bp. 160-200 ℃ at 100 mmHg; ^{31}P NMR ($CDCl_3$), 51.6 ppm.

Protected 2′-Deoxynucleosides. 5′-O-(4,4′-Dimethoxytrityl)-4-N-benzoyl-2′-deoxycytidine was synthesized using a modification of the reported procedures.[13] 3′-O-[(p-Chlorophenoxy)acetyl]thymidine was synthesized using the reported procedure.[3]

Synthesis of Protected R_p- and S_p- Diastereomers of d[Cp(s)T]. Following the reported procedure,[7,8] the phosphorylating agent (0.675 g, 3 mmol) was first allowed to react with 1-hydroxybenzotriazole (0.878 g, 6.5 mmol) and triethylamine (6 mmol) in tetrahydrofuran (4.8 mL) at room temperature. After 20 min, 5′-O-dimethoxytrityl-4-N-benzoyl-2′-deoxycytidine (1.268 g, 2 mmol) and dry pyridine (4 mL) were added to the reaction mixture. After a further reaction for 75 min, 3′-O-[(p-chlorophenoxy)acetyl] thymidine (0.822 g, 2 mmol) and pyridine (4 mL) were added and ensuing the reaction was allowed to proceed for 8 h before it was worked up. The saturated $NaHCO_3$ solution (4 mL) was added to the reaction mixture and the mixture was stirred for 10 min and extracted with chloroform (2×70 mL). Chloroform layer was washed with 0.1 M triethylammonium bicarbonate (TEAB) buffer solution, pH 7.5. The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The product was purified by short column chromatography over silica gel, eluting with a gradient of 2-10% ethanol in chloroform and analyzed by HPTLC plate. Yield, 0.636 g (25.1 %) for higher R_f isomer and 0.268 g (10.6%) for lower R_f isomer. R_f, 0.36 [$CHCl_3$-EtOH(95 : 5 v/v)] for fast moving isomer and R_f, 0.31 for slow moving isomer. ^{31}P NMR ($CDCl_3$) 63.12 ppm for higher R_f isomer and 62.13 ppm for lower R_f isomer. ^1H NMR (DMSO-d_6) δ 1.42 (s, 3H, CH_3), 2.25-6.60 (m, 4H, 2′-H), 3.72 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 4.1-4.6 (m, 4H, 4′-H, 5′-H), 4.90 (s, 2H, CO-CH_2-O), 5.80-5.95 (m, 2H, 3′-H), 6.18-6.30 (m, 2H, 1′-H), 6.8-7.75 (m, 25H), 8.0 (d, 2H), 8.2 (d, 1H), 11, 3 (bs, 1H, NH). Anal. Calcd for $C_{61}H_{55}N_5O_{15}P_1S_1Cl_3 \cdot 2H_2O$: C, 56.2; H, 4.56; N, 5.37. Found: C, 56.05; H, 4.50, N, 5.28.

Unblocking and purification of the fully protected Diastereomers of d[Cp(s)T]. The 3′-O-(p-chlorophenoxy)acetyl protecting group was removed by dissolving the product in dioxane (50 mL) and 25% aqueous ammonium solution and stirring at room temperature for 70 min. A solution of the protected diastereomer (0.0183 g, 0.02 mmol), syn-2-nitrobenzaldoxime (0.033 g, 0.20 mmol) and N^1, N^1, N^3, N^3-tetramethylguanidine (0.023 mL, 0.18 mmol) in dioxane-water (1 : 1 v/v, 0.6 mL) was stirred at room temperature.[14] After 18 h, the reaction mixture was concentrated under reduced pressure and the residue was redissolved in aqueous ammonia (d, 0.88, 5 mL) and stirred overnight at room temperature. After evaporation to dryness, 10 mL of water was added to it and bubbled CO_2 to adjust pH to 5. Aqueous solution was washed with $CHCl_3$ and diethyl ether. The aqueous layer was evaporated. The unblocked material was passed through a Sephadex G-15 and was chromatographed on DEAE-Sephadex A-25. The column was eluted with TEAE buffer (pH 7.5, linear gradient 0.001-1.0 M). ^{31}P NMR(D_2O) showed a peak at 56.42 ppm for higher R_f isomer and 55.92 ppm for lower R_f isomer, respectively.

Synthesis of 5′-O-DMTdC$^{Bz}{}_{p(S,DCPh)}$T-β-cyanoethyl-N, N-diisopropyl-aminophosphoramidite. 5′-O-DMTdC$^{Bz}{}_{p(S,DCPh)}$T (1.408 g, 1.30 mmol) was dried by repeated evaporation with THF (10 mL) and redissolved in THF (13 mL). To this, 3 equiv. of N,N-diisopropylethylamine (0.67 mL, 3.90 mmol) was added under nitrogen and stirred for 5 min at room temperature. 2 Equivalent of 2-cyanoethyl-N,N,-diisopropylaminochlorophophine (0.58 mL, 2.60 mmol) was added during a period of 5 min. The reaction mixture was then stirred for 2 h and was monitored by TLC. Then the solvent was evaporated *in vacuo*. The residue was poured into ethylacetate (50 mL), washed with 5% $NaHCO_3$ solution, saturated NaCl solution, dried with $MgSO_4$ and evaporated to a form. The crude product was dissolved in small volume of toluene and precipitated in petroleum ether at −20 ℃. Next the precipitate was purified by silica gel chromatography with a gradient of 1% Et_3N/hexane to 1% Et_3N/49% ethylacetate /hexane, followed by precipitation in petroleum ether at −20 ℃. The phosphoramidite was dried *in vacuo* and dissolved in dry acetonitrile to give 0.2 M solution which was used for automatic oligonucleotide synthesis.

Synthesis of Oligonucleotide. Oligonucleotides were synthesized using solid phase synthesis. The following synthesis cycle was used: (1) Wash with 1,2-dichloroethane (2× 2 mL); (2) detritylated by addition of 2 mL of 3% solution of trichloroacetic acid in 1,2-dichloroethane for 2 min; (3) wash with dichloromethane (3×2 mL); (4) render anhydrous by washing with acetonitrile (10×2 mL); (5) couple by addition of 100 mmol of the appropriate 5′-O-(dimethoxytrityl)nucleoside 3′-O-(morpholinomethoxyphosphite) in 0.5 mL of acetonitrile together with 250 mmol of tetrazole in 0.5 mL of acetonitrile (coupling times were 30 min for the first cycle and 10 min for subsequent cycles); (6) wash with acetonitrile (2×2 mL); (7) oxidize by addition of 1 mL of a 1% solution

of iodine dissolved in lutidine-THF-H_2O (1 : 8 : 1 v/v) for 1 min; (8) wash with acetonitrile (3×2 mL); (9) cap unreacted hydroxyl groups by addition of 1 mL of lutidine, and 0.25 mL of acetic anhydride for 5 min; (10) wash with acetonitrile (3×2 mL). Step 10 completes the addition of one nucleotide. The growing oligomer is further elongated by repeating the steps by beginning again at step 1. Phosphorothioate-containing oligomers were prepared by the addition of a chirally pure $DMTdC^{Bz}{}_{p(S,DCPh)}T_{pcpAc}$ dimer instead of a monomer. In this case, the only alteration in the protocol was an increase in the coupling time to 45 min. After the addition the last nucleotide the synthesis cycle was terminated with the completion of step 8. Place the dry support bearing the fully protected oligomer in a 25 mL round bottomed flask. The 2,5-dichlorophenyl group and the succinate linkage were cleaved by the treatment of a solution of 0.5 mL of syn-2-nitrobenzealdoxime (0.39 mmol) in dioxane and 44 mL of N^1,N^1,N^3,N^3-tetramethylguanidine (0.35 mmol). After 4 h, 0.73 mL of acetonitrile/water (4 : 1 v/v) was added and the flask was standed at room temperature for 20 h. followed evaporation. The base-protecting groups were removed by adding 5 mL of 28% aqueous ammonia and heating at 55 ℃ for 15 h. After this time the ammonia solution was removed by evaporation at a water pump. The product was dissolved in 3 mL of a 1% aqueous NEt_3 solution and silica gel removed by filtration through a small glass wool plug in a Pasteur pipette. The filtrate was extracted with ethyl acetate (3×3 mL), briefly evaporated at a water pump to remove excess ethyl acetate, and made up to about 1 mL. The dimethoxytrityl oligomer was purified by reverse-phase HPLC with a linear gradient of acetonitrile(from 5% to 50% in 25 min) in 0.1 M of triethyl ammonium acetate (pH 7.0), at 50 ℃. The dimethoxytrityl groups were then removed by a 1h treatment with 2 mL of 80% acetic acid. The acetic acid was removed by evaporation, the resulting oligomer was dissolved in 2 mL of water, and the solution was extracted with ethyl acetate (3×2 mL). Final purification, by injection of ten aliquots of 100 μL, was by reverse-phase HPLC with a linear gradient of acetonitrile (from 5% to 20% in 20 min) in 0.1 M of TEAA (pH 7.0). Fractions that contained product were pooled and evaporated to dryness. The purity of the oligomer was checked by reverse-phase HPLC. The purified oligomers were dissolved in 1 mL of sterilized water and stored frozen at −20 ℃.

Enzymatic Procedures. Snake venom phosphodiesterase. Hydrolysis of $d[C_{p(s)}A]$ was carried out at 37 ℃ in a system containing in a total volume of 200 μL, 100 mM Tris-HCl, pH 8.9, 100 mM NaCl, 14 mM $MgCl_2$, 0.2 mg dinucleotide, and 0.5 mg (0.21 u/mg) enzyme. At appropriate times 10 μL aliquots were quenched with 2 μL of ice-cold 1 M acetic acid and analyzed by HPLC.

Nuclease S1. $d[C_{p(s)}A]$ (0.2 mg) was dissolved in 200 μL of 100 mM Tris-HCl, pH 8.9, 100 mM NaCl, 14 mM $MgCl_2$ and digested with nuclease S1 (50 units) at 37 ℃. At appropriate times 10 μL aliquots were quenched with 2 μL of ice-cold 1 M acetic acid and analyzed by HPLC.

Restriction endonuclease XhoI. Oligonucleotide cleavage reaction with XhoI was performed by incubating oligomer (0.14 O.D.) at 37 ℃ in a system containing in a total volume of 200 μL of 6 mM Tris-HCl, pH 7.9, 90 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT and XhoI (20 units) overnight. For nucleotide containing phosphorothioate, amount of enyzme was increased to 100 unit and 100 μg/mL BSA was added to the reaction buffer.

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15. Abbreviations used are as follows: Bz, Benzoyl; DCPh, 2,5-dichlorophenyl; DMTr, 4,4′-dimethoxytrityl; DTT, dithiothreitol.