D. C.; Stauf, G. T.; Vaartstra, B. A.; Ostrander, R. L.; Rheingold, A. L. *Chem. Mater.* **1994**, *6*, 1967. (f) Kimura, T.; Yamauchi, H.; Machida, H.; Kokubun, H. *Jpn. J. Appl. Phys.* **1994**, *33*, 5119.
10. Perrin, D. D.; Armarego, L. F. *Purification of Laboratory chemicals*; 3rd ed.; Pergamon Press: Oxford, U.K., 1988.
11. (a) Moeller, T.; Kremers, H. E. *Chem. Rev.* **1945**, *37*, 114. (b) Pope, G. W.; Seinbach, J. F.; Wagner, W. F. *J. Inorg. Nucl. Chem.* **1961**, *20*, 304.
12. Philips, Jr., T.; Sands, D. E.; Wagner, W. F. *Inorg. Chem.* **1968**, *7*, 2295.
13. Berg, E. W.; Truemper, J. T. *J. Phys. Chem.* **1960**, *64*, 487.
14. (a) Richardson, M. F. *Inorg. Chem.* **1971**, *10*, 498. (b) Berg, E. W. *Anal. Chim. Acta* **1968**, *40*, 101. (c) *Gmelin Handbook Inorg. Chem. Syst.*; No 39, D3; 1981; 105.

Synthesis and Characterization of Dodecanucleotides Containing the XhoI Recognition Sequence with a Phosphorothioate Group at the Cleavage Site

Byung Jo Moon*, Sang Kook Kim, Nam Hee Kim, and Oh Shin Kwon

Department of Biochmistry, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea
Received July 12, 1996

The synthesis and characterization of diastreomeric dodecanucleotides, d[GATC$_{p(S)}$TCGAGATC], containing recognition sequence of the XhoI restriction endonuclease with a phosphorothioate internucleotidic linkage the cleavage site are described. R_p and S_p form of diastereomerically pure dinucleoside phosphorothioates d[C$_{p(S)}$T] were presynthesized and used for the addition to the growing oligonucleotide chain as a block. The stereochemistry of dinucleoside phosphorothioate was assigned by ^{31}P NMR spectroscopy, enzyme digestion, and reverse-phase HPLC. XhoI restriction endonuclease cut only R_p diastereomer d[GATC$_{p(S)}$TCGAGATC]. The rate of hydrolysis is slower than that of the unmodified dodecamer d[GATCTCGAGATC]. The phosphorothioate nucleotide is using for determination of the stereochemical course of the XhoI catalyzed reaction.

Introduction

Type II restriction endonucleases[1] catalyze the cleavage of double stranded DNA at sequence-specific sites typically 4-6 base pairs in length. Although these enzymes are immensely important in genetic engineering, not much mechanistic information is available.[2] Progress in understanding how these enzymes recognize and cut the sequence-specific sites of DNA has been aided by the crystal structures of both the EcoRI[3,4] and the EcoRV endonuclease.[5,6] Recent advances in the efficient synthesis of small oligonucleotides have made it possible to undertake a variety of mechanistic investigations with these enzymes. It has been known that certain restriction enzymes including EcoRI are capable of cleaving phosphorothioate internucleotidic lingkages when incorporated into the (−) strand of fd DNA, although at reduced rate. It is, therefore, possible to determine the streochemical course of the such an enzyme catalyzed reaction providing we could synthesize an oligonucleotide containing the appropriate recognition sequence with a phosphorothioate internucleotidic linkage of known absolute configuration at cleavage site. Restriction endonuclease catalyzed hydrolysis in the presence of $H_2{}^{18}O$ and subsequent nuclease P1 cleavage of the reaction products should furnish a deoxynucleoside 5'-[^{18}O]-phosphorothioate whose absolute configuration should be amenable to stereochemical analysis by ^{31}P NMR spectroscopy.[7] The knowledge of whether such an enzymatic reaction proceeds with retention or inversion of configuration at phosphorous provides evidence for or against the existence of a covalent enzyme intermediate and thus limits the number of mechanisms that can be proposed for an enzymatic reaction. Using phosphorothioate nucleotides, stereochemical course of reactions catalyzed by restriction endonucleases EcoRI[8] and EcoRV[9] were determined to be inversion of configuration at phosphorus, respectively. It is known that a structural homology between EcoRI[3], and EcoRV[10] in the vicinity of the phosphorus to be cleaved was noticed, consisting of four amino acids located in a similar steric arrangement. It was also demonstrated that the two acidic amino acids of this homologous region are important for catalysis. It was concluded, therefore, that the general mechanism of both enzymes is similar, including the same stereochemical course of reaction.[10]

We are interested whether other restriction endonucleases catalyze reactions with inversion of configuration at phosphorous as EcoRI and EcoRV did. We wish to describe here the synthesis and characterization of diastreomeric dodecanucleotides, d[GATC$_{p(S)}$TCGAGATC] (Figure 1), which con-

*To whom correspondence should be addressed.

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

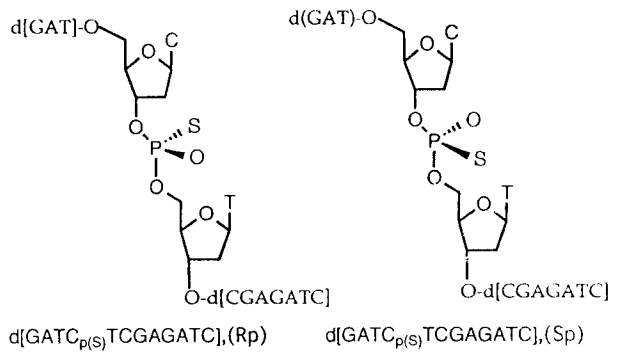

Figure 1. Configration of the S_p- and R_p- diastereomers of d [GATC$_{p(S)}$TCGAGATC].

tain recognition sequence of the XhoI restriction endonuclease[11] with a phosphorothioate internucleotidic linkage the cleavage site and could be used to study stereochemical course of reactions catalyzed by XhoI restriction endonuclease.

Materials and Methods

Materials. Nucleosides were obtained from Sigma Chemical Company. 2,5-Dichlorophenol, PCl_3 and $PSCl_3$ were purchased from Fluka Chemical Co. (Switzerland). Restriction endonuclease XhoI, Nuclease and P1 were purchased from Promega. Nuclease S1 was purchased from KOSCO and snake venom phosphodiesterase from Sigma Chemical Company. Pyridine, tetrahydrofuran, acetonitrile were distilled over calcium hydride. Dioxane was distilled over Na.

Purification Methods and Analytical Methods. Thin layer chromatography was performed on Merck Kiesel $60F_{254}$ plates and HPTLC plates that were eluted with chloroform methanol mixture. 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 absorbance detector (model 783A) and with gradient pump system (model 400) was employed. In all cases, the reverse phase Jones ODS column (5 μm particle size, 250×4.6 mm) was utilized. 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).

Synthetic Procedures

2,5-Dichlorophenylphosphorodichloridothioate. Following the procedure,[12] 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 10 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.[7]

Synthesis of Protected Rp- and Sp- Diastereomers of d[Cp(s)T]. Following the reported procedure,[14,15] 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-(4,4'-Dimethoxytrityl)-4-N-benzoyl-2'-deoxycytidine (1.268 g, 2 mmol) and dry pyridine (4 mL) were added to the resulting products. After a further period of 75 min, 3'-O-[(*p*-Chlorophenoxy)acetyl]thymidine (0.822 g, 2 mmol) and pyridine (4 mL) were added and ensuing 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 triethylammonium bicarbonate (TEAB) buffer solution (0.1 M, pH 7.5). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure.

Separation 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. After the 3'-OH protecting group was unblocked, the partially protected diastereomers of d[Cp(s)T] was purified and separated by short column chromatography over silica gel eluting with ethanol in chloroform and analyzed by HPTLC plate. Yield, 0.636 g (45.1%) 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.[16] 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 film, 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 TEAB buffer (pH 7.5, linear gradient 0.001-1.0 M). ^{31}P NMR (D_2O) 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-diisopropylaminophosphoramidite. 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 dichloromethane (2×2 mL); (2) detritylated by addition of 2 mL of 3% solution of trichloroacetic acid in dichloromethane 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-β-cyanoethyl-N,N-diisopropylaminophosphoramidite in 0.5 mL of acetonitrile together with 250 μmol 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 $DMTrC^{Bz}{}_{p(S,DCPh)}T$ 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-tetramethyl- guanidine (0.35 mmol). After 4 h, 0.73 mL of acetonitrile/water (4 : 1 v/v) was added and the flask was stood at room temperature for 20 h. followed evaporation. The baseprotecting 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 Et_3N 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 1 h 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 ℃.

Sequencing of Oligonucleotides. Oligonucleotides sequencing was performed by the chemical method of Maxam and Gilbert.[17]

Enzymatic Procedures

Nuclease P1. $d[C_{p(s)}T]$ (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 P1 (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 enzyme was increased to 100 unit and 100 μg/mL BSA was added to the reaction buffer.

DNA melting

Melting curves were obtained by measuring the change in absorption at 260 nm in a Shimadzu UV-265 spectrophotometer. The temperature was increased from 10 ℃ to 86 ℃ at a rate of 15 ℃/h using a TB-85 Thermo Bath Shimadzu circulating bath temperature controller. Samples were heated in a masked 1cm path length quartz cuvettes fitted with teflon stopper. Each thermal denaturation was performed in 10 mM Tris-HCl buffer, pH 7.2, 100 mM NaCl and 10 mM $MgCl_2$ at 0.5 O.D. of each strand. The sample was kept at 90 ℃ for 5 min and cooled to 10 ℃. At temperature below 20 ℃, N_2 gas was continuously passed through the sample compartment to prevent formation of condensate.

Results and Discussion

The dodecanucleotides containing a phosphorothioate group were synthesized by the phosphite method on a solid support.[18,19] Phosphorothioate containing oligomers can be prepared by the addition of elemental sulfur to the phosphite intermediate, resulting in a mixture of diastereomers of phosphorothioate oligomer. But it seems that separation of the diastereomers depends on the sequence of oligomers prepared. We could not separate certain diastereomers. Therefore, in order to prepare chirally pure oligomers, we have utilized use of a presynthesized diastereomerically pure phosphorothioate dimer as a building block. The desired dinucleoside phosphorothioate $d[C_{p(s)}T]$ was prepared by the phosphotriester approach.[20] The phosphorylating agent, 2,5-dichlorophenylphosphorodichloridothioate was prepared following modifications of the published procedure[12] in 72% yield. The $d[C_{p(s)}T]$ dimer was prepared by condensing by 5′-O-dimethoxytrityl-4-N-trimethylacetyl-2′-deoxycytidine and 3′-O-[(*p*-chlorophenoxy) acetyl] thymidine using 2,5-dichlorophenylphosphorodichloridothioate and 1-hydroxybenzotriazole as condensing agent in THF. Purification and separation of diastereomers were simultaneously achieved by short column chromatography over silica gel eluting with ethanol in chloroform.

It is important to note that before column chromatography removal of the (*p*-chlorophenoxy)acetyl protecting group was required. It is believed that the large and very hydrophobic character of the (*p*-chlorophenoxy)acetyl protecting group makes the separation difficult. After chromatography, the diastereomers were checked with high performance thin layer chromatography (HPTLC) plates because the difference in Rf values of two diastereomers were too small to be separated on regular TLC plates. Short column chromatography afforded the pure higher R_f [0.35, $CHCl_3$-EtOH (95 : 5 v/v)] diastereomer in 27% yield and the pure lower R_f (0.34) diastereomer in 18% isolated yield. ^{31}P NMR spectroscopy of the diastereomers at 25 ℃ in $CDCl_3$ showed resonance at 63.07 and 62.76 ppm, respectively.[18] ^{31}P NMR spectroscopy shows that each diastereomer was chirally pure without contamination with the other diastereomer. Their designation of configuration at phosphorus as R_p and S_p follows from analysis of ^{31}P NMR spectroscopy and reverse-phase HPLC and the stereospecificity in the hydrolysis catalyzed by nuclease P1. 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 56.26 and 55.68 ppm, respectively. Since it is known that the Sp diastereomer of dinucleotide phosphorothioates resonates at higher field than the R_p diastereomer,[14,21,22] this established the higher R_f contained the diastereomer with the S_p configuration. Confirmation of this results comes from reverse-phase HPLC analysis of the unblocked mixture in which the higher R_f diastereomer elutes before the lower one.[14] Again the R_p diastereomer of dinucleotide phosphorothioates is known to elute before the S_p in reverse-phase HPLC system.[21,22] The above results were confirmed by studying the hydrolysis of these diastereomers catalyzed by nuclease P1, which is known to hydrolyzes dinucleoside phosphorothioate of the S_p but not R_p configuration.[23] The results clearly demonstrate that the fast-moving isomer (higher R_f) of triester corresponds to the dinucleoside phosphorothioate of the R_p configuration. Diasteromers of dodecamers d[GATC$_{p(s)}$TCGAGATC] (Figure 1) which contain the recognition sequence for XhoI with a phosphorothioate group at the cleavage site were synthesized using the protected optically pure (R_p) and (S_p) diastereomers. First, the protected optically pure (R_p) and (S_p) diastereomers, DMTrdC$^{Bz}_{p(S,DCPh)}$T was converted to phosphoramidite to use as a building block in the phosphite method on a solid support. The β-cyanoethyl-N,N-diisopropylaminophosphoramidite dimers could be added to growning chain of oligmer in the usual fashion. The coupling time was increased to 45 min from 10 min. After completion of the solid phase synthesis the 2,5-dichlorophenyl group and the succinate linkage were cleaved by the treatment of syn-2-nitrobenzealdoxime in dioxane and N^1,N^1,N^3,N^3-tetramethylguanidine. Aqueous ammonia treatment removed the base-protecting groups and cleaved simultaneously the oligonucleotide from the solid support. The dimethoxytrityl oligomer was purified by reverse-phase HPLC with a linear gradient of acetonitrile. All the truncated sequence by-products resulting from incomplete coupling elute much earlier than the dodecamer on reverse-phase HPLC. The dimethoxytrityl groups were then removed by treatment with 80% acetic acid to give fully deblocked oligmer. Final purification of the oligomer was done by reverse-phase HPLC with a linear gradient of acetonitrile in TEAA. 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 ℃. The purity of the oligomers synthesized has been checked by reverse-phase HPLC and appeared over 95% pure. No separation was achieved between the S_p and R_p diastereomers of d[GATC$_{p(s)}$TCGAGATC] and both diastereomers elute later than normal oligonucleotide d[GATCTCGAGATC] on reverse-phase HPLC.

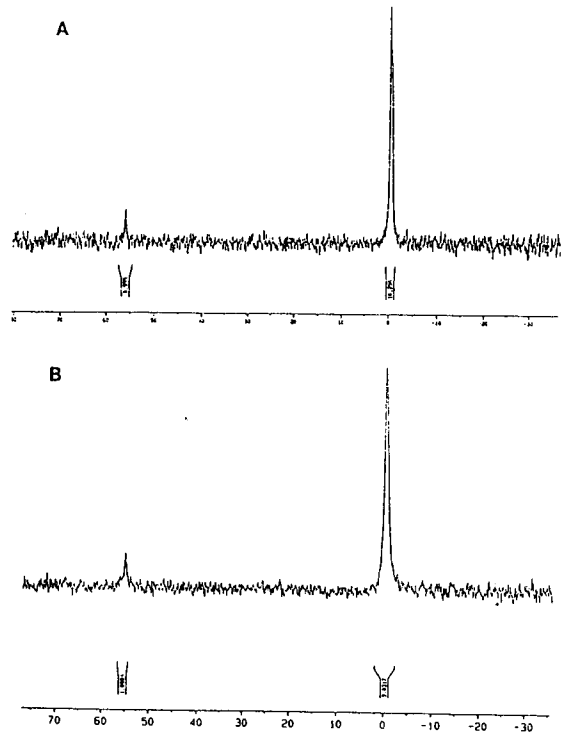

Figure 2. ^{31}P NMR spetra of the distereomers of the S_p- and R_p- diastereomers of d[GATC$_{p(s)}$TCGAGATC] at 10 ℃. The phosphorothioates group of S_p- and R_p- diastereomers resonate at δ 55.97 and 55.09(B), respectively. Two resonances at δ −0.29 and 55.97 of the spectrum of R_p isomer(A) and two resonances at δ −0.12 and 55.09 of the spectrum of S_p isomer (B) were in an approximate ratio of 10 : 1, respectively.

While analyzing the ^{31}P NMR spectroscopy of the R_p and S_p isomers of d[GATC$_{p(s)}$TCGAGATC] (Figure 2), it became apparent that there are distinct differences in the resonance of the phosphorothioate groups of diastereomers. ^{31}P NMR spectra of R_p and S_p isomer taken at 10 ℃ show a group of resonances at δ −0.29 and −0.12, respectively. The spectra of both R_p and S_p isomers show in addition signal at δ 55.97 and 55.09, respectively, representing the resonance of the phosphorothioate groups. Two resonances at δ −0.29 and 55.97 of the spectrum of R_p isomer and two resonances at δ −0.12 and 55.09 of the spectrum of S_p isomer were in an approximate ratio of 10 : 1, respectively, confirming that there are ten phosphate groups and one phosphorothioate

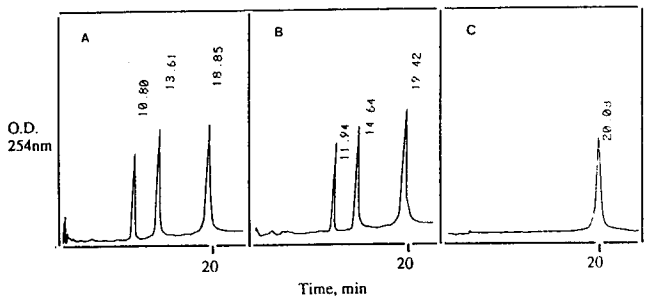

Figure 3. HPLC analysis of a partial XhoI digestion of oligonucleotide containing XhoI recognition sequence. (A) Parent oligonucleotide, GATCTCGAGATC, (B) R_p-GATC$_{p(s)}$TCGAGATC, and Sp-GATC$_{p(s)}$TCGAGATC. In all cases undigested oligomers elute latest.

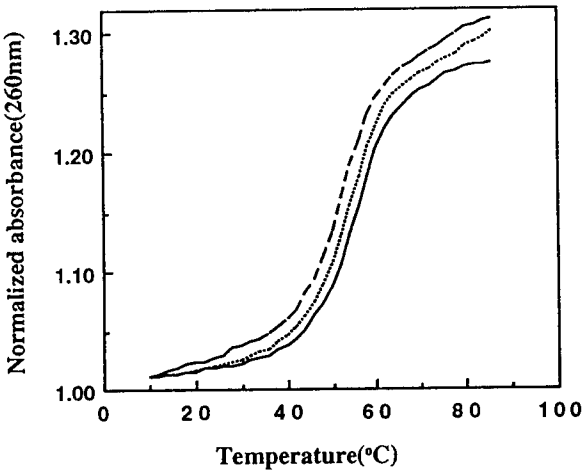

Figure 4. Thermal melting curves of oligonucleotides containing a phosphorothioate group compared to the melting curve of the parent oligomer. —; GATCTCGAGATC (Tm, 54.85 ℃), ···; R_p-GATC$_{p(s)}$TCGAGATC (Tm, 52.75 ℃), ---; S_p-GATC$_{p(s)}$TCGAGATC (Tm, 51.75 ℃).

group in each dodecaoligonucleotide.

As expected, d[GATCTCGAGATC] and the R_p diastereomers of d[GATC$_{p(s)}$TCGAGATC] were substrates for the XhoI restriction endonuclease. d[GATCTCGAGATC] was digested by XhoI to give only two products as monitored by HPLC (Figure 3). Collection of these products and analysis using the nuclease P1 and alkaline phosphatase treatment showed that these products were d[GATC] and d[$_p$TCGAGATC]. Treatment of the R_p diastereomers of d[GATCsTCGAGATC] with XhoI also gave only two products, d[GATC] and d[$_{p(S)}$TCGAGATC] as monitored by HPLC. The phosphorothioate dodecamer was cleaved approximately 8 times more slowly than the unmodified dodecamer.

A very important characteristic of the phosphorothioate dodecamers is their thermal stability. XhoI as well as other type II restriction endonucleases requires a double-stranded oligodeoxynucleotide as substrate. Figure 4 shows the thermal melting curves of the oligonucleotide duplexes. The solution had similar absorbances at 10 ℃ ($A_{1cm,260nm}$=0.5) and melting profiles of oligonucleotides are not so sensitive to sulfur substitution. Melting point of the R_p form of d[GATC$_{p(s)}$TCGAGATC] was lower than that of the unmodified dodecamer by 2 ℃, but slightly higher than that of the S_p diastereomer. This is significant since a decreased thermal stability has been reported for the phosphorothioate analogues of the alternating polynucleotides poly[d(G-C)] and poly[d(A-T)].[24,25] In these polymers thermal stability is lowed to the greatest extent when the pyrimidine nucleoside 5′-phosphate is substituted by a phosphorothioate. It has been reported that a decrease in T_m of 8 ℃ and 15 ℃ were observed for poly[d[$_pG_{p(S)}C$]] and poly[d[$_pA_{p(S)}T$]] respectively, whereas for the polymers containing a purine nucleoside 5′-phosphorothioate such as poly[d[$_pC_{p(S)}G$]] and poly[d[$_pT_{p(S)}A$]] the T_m values were lowed by 2 and 5 ℃, respectively . Contrary to this it was reported that the T_m values of octamer d[GG$_{p(s)}$AATTCC] was similar to that of d[GGAATTCC].[26] The reported results and our results indicate that single site sulfur substitution does not affect melting profiles of oligonucleotides not much. The sequence of oligonucleotides was confirmed by the chemical degradation method by Maxam-Gilbert. Maxam-Gilbert method gave the desired sequence and was not interrupted by phosphorothioate.

We are at present using the R_p form of d[GATC$_{p(s)}$TCGAGATC] (500O.D.) to evaluate the stereochemical course of the XhoI restriction endonuclease-catalyzed reaction.

Acknowledgment. This work was supported by a grant from Korea Science and Engineering Foundation through Center for Biofunctional Molecules and a grant from the Genetic Engineering Research Program, the Korea Ministry of Education.

References

1. Bennet, S. P.; Halford, S. E. *Current Topic in Cell Regulation* **1989**, *30*, 57.
2. Modrich, P. *CRC Crit. Rev. Biochem.* **1982**, *13*, 2878.
3. Kim, Y.; Grable, J. C.; Love, R.; Greene, P. J.; Rosenberg, J. M. *Science* **1990**, *249*, 1307.
4. Rosenberg, J. M. *Current Opinion in Structural Biology*, **1991**, *1*, 104.
5. Winkler, F. K. *Current Opinion in Structural Biology* **1992**, *2*, 93.
6. Kostrewa, D.; Winkler, F. K. *Biochemistry* **1995**, *34*, 683.
7. Eckstein, F. K. *Angew. Chem.* **1983**, *95*, 431.
8. Connolly, B.; Eckstein, F.; Pingoud, *J. Biol. Chem.* **1984**, *259*, 10760.
9. Grasby, J. A.; Connolly, B. A. *Biochemistry*, **1992**, *31*, 7855.
10. Selent, U.; Ruter, T.; Kohler, E.; Liedtke, M.; Thielking, V.; Alves, J.; Oelgeschlager, T.; Wolfes, H.; Peter, F.; Pingoud, A. *Biochemistry* **1992**, *31*, 4808.
11. Gingeras, T. R.; Myers, P. A.; Olson, J. A.; Hanberg, F. A.; Roberts, R. J. *J. Mol. Biol.* **1978**, *118*, 113.
12. Tolkmith, H. *J. Org. Chem.* **1958**, *23*, 1682.
13. Jones, R. *in Oligonucleotide Synthesis*; Gait, M.(ed), **1984**, IRL Press, p. 23.
14. Kemal, O.; Reese, C. B.; Serafinowska, H. *J. Chem. Soc., Chem. Commun.* **1983**, 591.
15. Moon, B. J.; Huh, K. L.; Kim, S. K. *Bull. Korean Chem. Soc.* **1993**, *14*, 52.
16. Reese, C. B.; Titmus, R.; Yau, L. *Tet. Lett.* **1978**, 2727.
17. Maxam, A. M.; Gilbert, W. *Proc. Natl. Acad. Sci.* **1977**, *74*, 560.

18. Matteucci, M. D.; Caruthers, M. H. *J. Am. Chem. Soc.* **1981**, *103*, 3185.
19. McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 245.
20. Moon, B. J.; Jeong, H. J.; Kim, S. K.; Kim, N. H. *Bull. Korean Chem. Soc.* **1996**, *17*, 24.
21. Romaniuk, P.; Eckstein, F. *J. Biol. Chem.* **1982**, *257*, 7684.
22. Bartlett, P.; Eckstein, F. *J. Biol. Chem.* **1982**, *257*, 8878.
23. Gaffrey, B.; Jones, R.; Ti, G. S. *J. Am. Chem. Soc.* **1982**, *104*, 1326.
24. Ecckstein, F.; Jovin, F. M. *Biochemistry* **1983**, *22*, 423.
25. Jovin, T. M.; van der Sane, J. H.; Zarling, D. A.; Arndt-Jovin, D. J.; Eckstein, F. Fuldner, H. H.; Greider, C.; Grieger, I.; Hamori, E.; Kalish, B.; McIntosh, L. P.; Robert-Nicoud, M. *Cold Spring Habor Sym. Quant. Biol.* **1983**, *47*, 143.
26. Connolly, B. A.; Potter, B. V. L.; Eckstein, F.; Pingoud, A.; L. Grotjahn, *Biochemistry* **1984**, *23*, 3443.

Characterization of Extremely Hydrophobic Immunostimulatory Lipoidal Peptides by Matrix Assisted Laser Desorption Ionization Mass Spectrometry

Jung-Suk Jang†, Sung-Taek Lee‡, and Yoon-Seok Chang*

**Department of Chemistry, Hanyang University, Ansan 425-791, Korea*
†*Korea Research Institute of Chemical Technology, Taejon 305-606, Korea*
‡*Department of Biological Science, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea*
Received July 24, 1996

Synthetic lipoidal peptides based on viral protein sequences have been prepared. These peptides contain an N-palmitoyl group at the N-terminal residue, which is a modified cysteine, containing a S-[2,3-bis(acyloxy)-(2-R,S)-propyl] moiety. When this residue (Pam_3Cys) is at the N-terminus of a synthetic peptide, it acts as potent immunoadjuvant to enhance both IgM and IgG antibody responses to the attached peptide. Conventional analytical procedures (*e.g.*, Edman degradation and amino acid analysis) are either not applicable due to the N-terminal modification, or do not provide confirmation of the intact structure. Chromatographic analysis is also hindered by the tendency of these lipoidal Pam_3Cys peptides to form large aggregates, and in some cases to be permanently adsorbed on reversed phase columns. We have applied several mass spectrometric techniques, including fast atom bombardment (FAB), electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) to characterize the intact structures of a number of different Pam_3Cys synthetic peptides. The MALDI-MS has been found to be the most sensitive for the analysis of the structure of Pam_3Cys peptides.

Introduction

Synthetic peptides are prepared to contain an N-palmitoyl moiety at the N-terminal residue of the peptide which is a modified cysteine, containing a S-[2,3-bis(acyloxy)-(2-R,S)-propyl] moiety. When this residue is placed at the N-terminus of various synthetic peptides, it has been found to be potent immunoadjuvant which enhances both IgM and IgG antibody responses to the attached peptide.[1~6] Synthetic analogues of these compounds include those bearing palmitoyl groups (Pam_3Cys) as shown in Figure 1. These synthetic peptides have significant advantages, since the addition of other adjuvants are not required, and most importantly, the epitope can be specifically defined.

It is critical, however, that these peptides should be structurally characterized prior to their use in immunological studies.[7~8] This is most important, since the synthesis involves several steps where the peptide is exposed to conditions that can provide amino acid side chain modification and/or deacylation. And, the lipoidal nature of the peptides make them extremely difficult to be purified and analyzed. Reverse phase HPLC can lead to irreversible adsorption to the bonded phase. Although we have found that the N-methyl-2-pyrrolidone (NMP) is useful for solubilization and isocratic elution for some of these lipopeptides, it is not successful in all cases for HPLC purification.[9] The peak broadening resulting from the inherent self-aggregation of these compounds may, in even favorable cases, obscure contaminating peaks. Thus, amino acid analysis is not adequate to fully characterize these peptides prior to their use in immunological studies. Further, because the N-terminal is blocked, traditional Edman sequencing cannot be employed to determine the proper sequence of synthetic peptide.

We are currently using several mass spectral techniques to characterize the amino acid sequences of the Pam_3Cys peptides found in the envelop glycoproteins of HIV-1 and the Simian Immunodeficiency Virus (SIV).[17] Conventional FAB-MS analysis using standard matrices, such as glycerol and nitrobenzyl alcohol, is not particularly effective for these molecules, largely due to their tendency to aggregate. Here,