Synthesis and Characterization of an Octanucleotide Containing the *Eco*RI Recognition Sequence with a Phosphorothioate Group at the Cleavage Site†

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ABSTRACT: The synthesis and characterization of an octanucleotide, d(GGsAATTCC), containing the recognition sequence of the *Eco*RI restriction endonuclease with a phosphorothioate internucleotidic linkage at the cleavage site are described. Two approaches for the synthesis of the R_P and S_P diastereomers of this octamer by the phosphite method are presented. The first consists of the addition of sulfur instead of H_2O to the phosphite at the appropriate position during chain elongation. This method results in a mixture of diastereomers that can be separated by high-performance liquid chromatography after 5′-terminal phosphorylation. The second uses the presynthesized and diastereomerically pure dinucleoside phosphorothioate d[Gp(S)A] for the addition to the growing oligonucleotide chain as a block. The products are characterized by digestion with nuclease P1, fast atom bombardment mass spectrometry, ^{31}P NMR spectroscopy, and conversion to d(GGAATTCC) by desulfurization with iodine. Only the R_P diastereomers of d(GGsAATTCC) and its 5′-phosphorylated derivative are cleaved by *Eco*RI endonuclease. The rate of hydrolysis is slower than that of the unmodified octamer. The phosphorothioate octamer will be useful for the determination of the stereochemical course of the *Eco*RI-catalyzed reaction.

Restriction endonucleases catalyze the cleavage of double-stranded DNA at sequence-specific sites. Although these enzymes are immensely important in genetic engineering, little mechanistic information is available [see review by Modrich (1982)]. The recent advances in the efficient synthesis of small oligonucleotides have made it possible to undertake a variety of mechanistic investigations with these enzymes. We had observed earlier (Vosberg & Eckstein, 1982) that certain restriction enzymes including *Eco*RI (B. V. L. Potter, H. P. Vosberg, and F. Eckstein, unpublished results) are capable of cleaving phosphorothioate internucleotidic linkages when incorporated into the (−) strand of fd DNA, although at reduced rates. This suggested to us that it should be feasible to determine the stereochemical course of such an enzyme reaction providing we could synthesize an oligonucleotide containing the appropriate recognition sequence with a phosphorothioate internucleotidic linkage of known absolute configuration at the cleavage site. 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 [see review by Eckstein (1983a,b)]. The knowledge of whether such an enzymatic reaction proceeds with retention or inversion of configuration at phosphorus 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. We wish to report here the successful synthesis and characterization of the octanucleotide d(GGsAATTCC), which contains the recognition sequence for the restriction endonuclease *Eco*RI and a phosphorothioate group at the cleavage site. The determination of the stereochemical course of the reaction catalyzed by this enzyme using this octamer will be reported at a later date.

Materials and Methods

Nucleosides were obtained from Pharma-Waldhof (Düsseldorf, West Germany). Benzoyl chloride, anisoyl chloride, and isopropionyl chloride were purchased from EGA Chemie (Steinheim, West Germany) and were redistilled before use. 1*H*-Tetrazole was a product of EGA Chemie and was purified by sublimation at 100 °C and 0.05 mmHg prior to use. Pyridine, 2,6-lutidine, and *N*-ethyldiisopropylamine were purchased from Merck (Darmstadt, West Germany) and were refluxed with and then distilled from calcium hydride and stored over 4-Å molecular sieves. Acetonitrile used in the solid-phase nucleotide synthesis was an HPLC grade reagent from J. T. Baker Chemicals (Deventer, Holland). It usually contains 0.01% water but was stored over 4-Å molecular sieves and otherwise used as supplied. THF[1] used in the solid-phase synthesis was Merck dried reagent (maximum H_2O content 0.01%) as was Me_2SO (maximum H_2O content 0.03%) used to prepare phosphorothioate-containing dimers. These solvents were also stored over 4-Å molecular sieves. All other solvents used in the preparation of oligonucleotides were p.a. grade and were stored over 4-Å molecular sieves. Nuclease P1 (200 units/mg) was obtained from Sigma (Munich, West Germany), and alkaline phosphatase (from calf intestine, 2500 units/mg, molecular biology grade) was purchased from Boehringer Mannheim (West Germany). Polynucleotide kinase (from T4 infected *E. coli*, 5 units/μL) was a product

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[1] Abbreviations: FAB, fast atom bombardment; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; THF, tetrahydrofuran; Me_2SO, dimethyl sulfoxide; TEAA, triethylammonium acetate; TEAB, triethylammonium bicarbonate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; (R_P)- and (S_P)-d[Gp(S)A], R_P and S_P diastereomers of 5′-*O*-(2′-deoxyadenosyl) 3′-*O*-(2′-deoxyguanosyl) phosphorothioate; dAMPS, 2′-deoxyadenosine 5′-*O*-phosphorothioate; (R_P)- and (S_P)-d(GGsAATTCC), the R_P and S_P diastereomers of the octamer d(GGAATTCC) containing a d[Gp(S)A] unit instead of d(GpA); d(pGGsAATTCC), the 5′-phosphorylated octamer; $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{mmp}$, 5′-*O*-[$N^6$-benzoyl-3′-*O*-(morpholinomethoxyphosphino)-2′-deoxyadenosyl] 3′-*O*-[N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosyl] *O*-methyl phosphorothioate; $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{OH}$, 5′-*O*-($N^6$-benzoyl-2′-deoxyadenosyl) 3′-*O*-[N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosyl] *O*-methyl phosphorothioate; d, dalton.

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of P-L Biochemicals (St. Goar, West Germany). Tetrabutylammonium hydroxide used for HPLC was obtained from Waters Associates (Milford, MA) under the name PIC Reagent A. KH_2PO_4 (Merck, p.a. grade) used for HPLC was further purified by passage over Chelex resin to remove UV-absorbing impurities (Karkas et al., 1981). All other reagents were of the best quality available and were used as received. TLC was performed with silica gel 60 F_{254} plates (Merck, Darmstadt, West Germany). *Eco*RI endonuclease was isolated from an *Eco*RI overproducing strain kindly provided by Dr. M. Zabeau (Heidelberg) and was purified by chromatography on phosphocellulose and DEAE-cellulose as described (Langowski et al., 1980).

Methoxydichlorophosphine was prepared as described (Martin & Pizzolato, 1950). Methoxymorpholinochlorophosphine was synthesized by the procedure of McBride & Caruthers (1983) using *N*-(trimethylsilyl)morpholine (Pike & Schauch, 1962) as starting material. 5′-*O*-(Dimethoxytrityl)thymidine, N^6-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyadenosine, and N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine were synthesized by the procedures of Schaller et al. (1963) as modified by Gait et al. (1982a). N^4-Anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine was prepared by the method of Schaller et al. (1963). All these compounds were purified by flash chromatography (Still et al., 1978) on silica gel 60 (Merck, particle size 0.040–0.063 mm) using $CHCl_3$–CH_3OH mixtures under a positive nitrogen pressure of 0.5 atm. All dimethoxytrityl derivatives appeared pure by TLC using either $CHCl_3$–CH_3OH (95:5 v/v) or ethyl acetate–CH_3OH (95:5 v/v) as solvent. Protected deoxyribonucleoside morpholinomethoxyphosphites were synthesized by reacting the appropriate base-protected dimethoxytrityl nucleosides with methoxymorpholinochlorophosphine. The reaction conditions and purification protocols given by Dörper & Winnacker (1983) were followed with the exception that CH_2Cl_2 instead of $CHCl_3$ was used as the reaction solvent. After purification, the protected nucleoside methoxymorpholinophosphites appeared pure as judged by TLC using either $CHCl_3$–EtOAc–NEt_3 (45:45:10 v/v) or $CHCl_3$–CH_3OH–NEt_3 (85:5:10 v/v) and ^{31}P NMR spectroscopy (T derivative, δ 144.85 and 145.10; all other derivatives, δ 145.1 and 145.4 for the two diastereomers). Silica gel used as the solid support in oligonucleotide synthesis was Fractosil 200 (Merck, Darmstadt, West Germany) and was functionalized so as to possess free amino groups by the procedure of Caruthers (1982). N^4-Anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine was attached via the 3′-hydroxyl group to this amino silica gel as reported by Caruthers (1982). A loading of 113 μmol of N^4-anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine per gram of resin was achieved.

HPLC was performed with two Waters Associates Model 6000 A pumps controlled by a Model 660 solvent programmer. In all cases the reverse-phase octadecyl material ODS-Hypersil [5-μm particle size, supplied by Gynkotek (München, West Germany)] was utilized as the stationary phase although the buffers used for the mobile phase varied with the particular application. For the purification of dimethoxytrityl oligonucleotides, a linear gradient (flow rate 6 mL min^{-1}) consisting of 100 mM TEAA, pH 7 (A), and 100 mM TEAA, pH 7, containing 70% CH_3CN (B) was used (t = 0 min, 20% B; t = 20 min, 80% B) (gradient I). To purify completely deblocked oligonucleotides, a linear gradient (flow rate 3.5 mL min^{-1}) consisting of 100 mM TEAB, pH 8 (A), and 100 mM TEAB, pH 8, containing 60% CH_3CN (B) was used (t = 0 min, 5% B; t = 20 min, 30% B) (gradient II). This buffer system was further used, both to monitor the reactions and to purify the products of (1) the *Eco*RI-catalyzed hydrolysis of the various octanucleotides, (2) the desulfurization of phosphorothioate-containing oligomers with iodine, and (3) the 5′-phosphorylation of octanucleotides with polynucleotide kinase. The purity of the oligonucleotides was checked by using three systems. These all consisted of a 20-min linear gradient (flow rate 2 mL min^{-1}) produced from (1) 100 mM TEAA, pH 7.0 (A), and 100 mM TEAA, pH 7.0, containing 60% CH_3CN (B) (t = 0 min, 5% B; t = 20 min, 30% B) (gradient III), (2) 5 mM tetrabutylammonium hydroxide, pH 7.5, containing 4% CH_3CN (A) and 5 mM tetrabutylammonium hydroxide, pH 7.5, containing 70% CH_3CN (B) (t = 0 min, 30% B; t = 20 min, 80% B) (gradient IV), and (3) 50 mM KH_2PO_4, pH 6 (A), and 50 mM KH_2PO_4, pH 6, containing 30% CH_3CN (B) (t = 0 min, 5% B; t = 20 min, 30% B) (gradient V). To resolve the nuclease P1 digestion products of the various oligonucleotides, an upward concave (Waters solvent programmer curve 9) gradient (flow rate 1.5 mL min^{-1}) prepared from 50 mM KH_2PO_4, pH 6.5 (A), and 50 mM KH_2PO_4, pH 6.5, containing 30% CH_3CN (B) was used (t = 0 min, 0% B; t = 15 min, 50% B) (gradient VI). To separate the nuclease P1–alkaline phosphatase codigestion products, a linear gradient (flow rate 2 mL min^{-1}) produced from 50 mM KH_2PO_4, pH 6 (A), and 50 mM KH_2PO_4, pH 6, containing 30% CH_3CN (B) (t = 0 min, 5% B; t = 20 min, 50% B) had to be used (gradient VII). Routinely, a column 25 cm long with an internal diameter of 0.4 cm was used, the only exception being in the purification of dimethoxytrityl oligonucleotides when these dimensions were 30 × 0.8 cm.

^{31}P NMR spectra were recorded on a Bruker WP200 SY spectrometer operating at 81.01 MHz with quadrature detection and ^1H broad-band decoupling. Samples were contained in 5-mm precision tubes containing a concentric capillary filled with the appropriate reference. Chemical shifts are given in parts per million and are positive when downfield from the standard. Samples soluble in organic solvents were recorded in $CDCl_3$ containing 2% pyridine, and aqueous samples (with the exception of the octanucleotides) were measured in 100 mM EDTA adjusted to pH 8 with NaOH and containing 50% D_2O. These samples were referenced to 85% H_3PO_4. The spectra of the octanucleotides were recorded in 25 mM Hepes–NaOH, pH 7.5, containing 25 mM EDTA, 50 mM NaCl, and 30% D_2O as solvent and trimethyl phosphate as standard. Samples of 1–2 μmol in a total volume of 400 μL were used.

Mass spectra were recorded on a Kratos MS 50S mass spectrometer with a Kratos FAB source in the negative ion mode. The atom gun used xenon and produced a beam of neutral atoms at 8–9 kV. An aqueous solution of the triethylammonium salt of the nucleotide (1–2 μL, containing approximately 20 nmol) was injected into the glycerol matrix (approximately 2 μL) present on the FAB copper probe tip. Water was removed in the direct insertion lock, and the spectra were recorded at a magnet scan rate of 300 s/decade.

Melting curves were recorded in 1-cm cuvettes in a Zeiss DMR 10 spectrophotometer to which a Colora WK5 cryothermostat was attached. The temperature was measured with a Knauer precision temperature bridge and a thermistor enclosed in a glass capillary that extended through the stopper of the cuvette into the solution. Differential melting curves were computed by taking small temperature intervals from the integral recording. All samples were examined at an optical density of between 0.2 and 0.3 at 260 nm in 20 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 10 mM $MgCl_2$.

(p-Chlorophenoxy)acetic Anhydride. (*p*-Chlorophenoxy)acetic acid (46.5 g, 0.25 mol) was dissolved in ethyl acetate (400 mL) and ether (150 mL), and dicyclohexylcarbodiimide (25 g, 0.12 mol) was added. The reaction mixture was stirred at room temperature for 2 h, the precipitated dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness. The crude product was recrystallized from ether (500 mL): yield 60%; mp 90–91 °C [lit. 90–91 °C (van Boom et al., 1971)].

N^6-Benzoyl-3'-O-[(p-chlorophenoxy)acetyl]-2'-deoxyadenosine. N^6-Benzoyl-5'-*O*-(dimethoxytrityl)-2'-deoxyadenosine (5 mmol, 3.62 g) was dissolved in 20 mL of pyridine, and (*p*-chlorophenoxy)acetic anhydride (6 mmol, 2.13 g) was added. The reaction was monitored by TLC [$CHCl_3$–CH_3OH (9:1 v/v); R_f of starting material 0.8, R_f of product 0.95] and was usually complete in 3 h. Occasionally a second addition (2 mmol) of the anhydride was needed to ensure complete reaction. When the reaction was finished, methanol (10 mL) was added and after a further 2 h the solvent was evaporated at a water pump. Pyridine was removed by successive coevaporations with toluene using an oil pump. The dimethoxytrityl group was removed by the addition of 100 mL of an ice-cold solution of 2% *p*-toluenesulfonic acid in $CHCl_3$–CH_3OH (7:3 v/v). After 5 min on ice the mixture was poured into 250 mL of 5% aqueous $NaHCO_3$. The chloroform layer was washed with a further 250 mL of 5% aqueous $NaHCO_3$, 250 mL of saturated NaCl, and 250 mL of water. The N^6-benzoyl-3'-*O*-[(*p*-chlorophenoxy)acetyl]-2'-deoxyadenosine remains in the chloroform layer during these extractions as a fine dispersion. Purification can be simply achieved by filtration and washing of the residue sequentially with water, chloroform, and ether. The product appeared pure by TLC [$CHCl_3$–CH_3OH (8:2 v/v)]: R_f 0.7; yield 90%.

R_P and S_P Diastereomers of 5'-O-(N^6-Benzoyl-2'-deoxyadenosyl) 3'-O-[N^2-Isobutyryl-5'-O-(dimethoxytrityl)-2'-deoxyguanosyl] O-Methyl Phosphorothioate. N^6-Benzoyl-3'-*O*-[(*p*-chlorophenoxy)acetyl]-2'-deoxyadenosine (1 mmol, 493 mg) was dissolved in 1 mL of Me_2SO and 5 mL of THF. N^2-Isobutyryl-5'-*O*-(dimethoxytrityl)-2'-deoxyguanosine 3'-*O*-(morpholinomethoxyphosphite) (1 mmol, 794 mg) was dissolved in 5 mL of THF and added to the solution of the deoxyadenosine compound. Tetrazole (4 mmol, 280 mg) was then added and the mixture set aside for 1 h; then it was poured into a suspension of elemental sulfur (320 mg, 10 mmol) in 10 mL of pyridine and stirred for a further 1 h. The sulfur was then removed by filtration through glass wool and the solution evaporated to an oil with a water pump. Excess pyridine was removed by coevaporation with toluene using an oil pump. The viscous liquid so obtained was dissolved in 50 mL of $CHCl_3$ and the solution extracted twice with 50 mL of 5% aqueous $NaHCO_3$ and twice with 50 mL of saturated NaCl. The $CHCl_3$ layer was dried over Na_2SO_4 and evaporated to dryness. At this stage TLC [$CHCl_3$–CH_3OH (9:1 v/v)] showed the product (R_f 0.8) as the main component together with unreacted deoxyadenosine starting material (R_f 0.7) and some trityl positive material at the origin as minor contaminants. The 3'-*O*-[(*p*-chlorophenoxy)acetyl] protecting group was removed by dissolving the crude product in dioxane (16 mL) and 25% aqueous NH_3 solution (4 mL) and setting it aside at room temperature for 90 min. The R_f value of the desired product in the above TLC system is 0.5. The final product was purified by passage over a column of silica gel 60 equilibrated with $CHCl_3$–CH_3OH–pyridine (95:4:1 v/v) and eluted with the same solvent under a positive nitrogen pressure of 0.7 atm. Fractions of 10 mL were collected and analyzed by TLC silica gel plates with concentrating zones, eluted with $CHCl_3$–CH_3OH–pyridine (92:7:1 v/v). Fractions 76–85 (fast diastereomer) and 87–97 (slow diastereomer) were pooled and evaporated to dryness. As detailed below, the fast and slow isomers have the *S* and *R* configurations at phosphorus, respectively. Both diastereomers appeared ≥95% in the above TLC system with R_f values of 0.4 and 0.3 for the fast and slow isomers, respectively. Additionally, both isomers were ≥95% pure by ^{31}P NMR spectroscopy. δ values of 69.67 and 69.86 were found for the fast and slow isomers, respectively, when measured in $CDCl_3$–pyridine (98:2 v/v). Each diastereomer was obtained in yields of between 25 and 35% (overall yield between 50 and 70%). In order to establish the absolute configurations of the two diastereomers, a mixture of the fast and slow isomers (1:3 equiv of each) was completely deblocked as follows. Approximately 25 mg of the mixture was dissolved in dioxane (200 μL), triethylamine (100 μL), and thiophenol (100 μL) and left at room temperature for 90 min. The dimer was then precipitated with petroleum ether and the precipitate triturated with 3 × 10 mL of petroleum ether to remove excess thiophenol. Solvent was removed by evaporation, the precipitate dissolved in 2 mL of 25% aqueous ammonia, and the solution heated at 50 °C for 5 h. The ammonia was removed under reduced pressure and the residue taken up in 500 μL of 80% acetic acid and set aside at room temperature for 1 h. The acetic acid was removed by several coevaporations with water, the product obtained was dissolved in 2 mL of water, and the solution was extracted 3 times with 5 mL of ether. The aqueous layer was evaporated to dryness and the residue dissolved in a small volume of water. Reverse-phase HPLC (gradient VI) of the product revealed two peaks in a ratio of 3:1 eluting at 2.5 and 4 min, respectively, which coeluted with a standard mixture of the two diastereomers of d[Gp(S)A]. The later-eluting small peak in the HPLC was completely digested by nuclease P1, giving dG and dAMPS, whereas the early large peak was not hydrolyzed by this enzyme. ^{31}P NMR spectroscopy of the completely deblocked dinucleoside phosphorothioate showed two resonances of 3:1 intensity at δ 55.88 and 54.86, respectively.

5'-O-[N^6-Benzoyl-3'-O-(morpholinomethoxyphosphino)-2'-deoxyadenosyl] 3'-O-[N^2-Isobutyryl-5'-O-(dimethoxytrityl)-2'-deoxyguanosyl] O-Methyl Phosphorothioate. $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{OH}$ (350 μmol, 350 mg, diastereomerically pure) was dissolved in 5 mL of CH_2Cl_2 (freshly passed over basic alumina to remove acidic contaminants) in a 10-mL flask, and diisopropylethylamine (1.4 mmol, 250 μL) was added. The flask was sealed with a rubber septum, flushed with dry nitrogen, and cooled to 0 °C. Morpholinomethoxychlorophosphine (700 μmol, 100 μL) was added with a syringe and the mixture left on ice for 30 min. Ethyl acetate (10 mL, prewashed with 5% $NaHCO_3$) was added and the mixture extracted with 10 mL of 5% $NaHCO_3$ followed by 10 mL of saturated NaCl. The organic phase was applied directly to a column (10 × 2.5 cm) of silica gel 60 (230–400 mesh) equilibrated with EtOAc–CH_3CN–NEt_3 (7:2:1 v/v), and products were eluted with this solvent under a positive nitrogen pressure of 1 atm. Fractions of 5 mL were collected, and those containing product (fractions 6–25) were pooled and evaporated to dryness. Yields of 70% were typically obtained, and the product appeared to be about 90% pure by TLC [$CHCl_3$–CH_3OH–NEt_3 (9:0.5:0.5 v/v) or EtOAc–CH_3CN–NEt_3 (7:2:1); R_f 0.75 in each case] and was used without further characterization.

Solid-Phase Oligonucleotide Synthesis. Oligonucleotides were synthesized in a 2-mL glass syringe fitted with a glass

frit (porosity of 3) and a 10 cm long needle (Tanaka & Letsinger, 1982). The syringe was charged with 90 mg of silica gel containing 10 μmol of bound N^4-anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine. The 5′-*O*-(dimethoxytrityl) 3′-(morpholinomethoxyphosphite) derivatives of N^4-anisoyl-2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, and N^2-isobutyryl-2′-deoxyguanosine were used to prepare the nucleotide chain. In all cases, additions to and expulsions from the syringe were made via the needle.

The following synthesis cycle was used: (1) Wash with 1,2-dichloroethane (2 × 2 mL); (2) detritylate by addition of 2 mL of 10% solution of trichloroacetic acid in 1,2-dichloroethane for 2 min; (3) wash with 1,2-dichloroethane (3 × 2 mL); (4) render anhydrous by washing with acetonitrile (10 × 2 mL); (5) couple by addition of 100 μmol of the appropriate 5′-*O*-(dimethoxytrityl) nucleoside 3′-*O*-(morpholinomethoxyphosphite) 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 a 10% solution of (dimethylamino)pyridine in THF, 0.25 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 beginning again at step 1.

Phosphorothioate-containing oligomers were prepared by two methods. The first method was the addition of elemental sulfur to the phosphite intermediate, resulting ultimately in a mixture of diastereomers of the phosphorothioate oligomer prepared. In this case, after nucleotide coupling (step 5) the silica gel was washed with THF (3 × 2 mL) and a suspension of elemental sulfur (100 mg) in pyridine (2 mL) added. This addition was made with a Pasteur pipet after removing the syringe piston. The piston was then replaced and the syringe and contents were gently shaken for 2 h. Excess pyridine was expelled and the sulfur removed by the uptake and expulsion of 2 mL of a 50:50 mixture of CS_2–pyridine. Elemental sulfur is soluble in this mixture and four cycles are enough to ensure its removal. The silica gel was then washed with pyridine (4 × 2 mL) and the synthesis cycle continued at step 8. The second method used to produce phosphorothioate oligomers was the addition of a chirally pure $DMTdG^{ib}p(S,OCH_3)dA^{bz}_{mmp}$ 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 of the last nucleotide the synthesis cycle was terminated with the completion of step 8. The methyl groups were removed from the phosphotriester in the syringe by the addition of 2 mL of dioxane–NEt_3–thiophenol (2:1:1 v/v) for 1 h. This solution was then expelled and the silica gel washed with methanol (3 × 2 mL) and then ether (3 × 2 mL). The syringe piston was removed and the silica gel dried by the careful passage of nitrogen (entry via the barrel, exit via the needle) through the gel bed. Thus dried, the silica gel was easily poured into a 25-mL round-bottomed flask. The oligomer was cleaved from the silica gel, and the base-protecting groups were simultaneously removed by adding 3 mL of 25% aqueous ammonia and heating at 50 °C for 15 h. After this time the ammonia solution was removed by evaporation at a water pump. Care should be taken with this step as this solution has a tendency to froth. The product was dissolved in 1 mL of a 1% aqueous NEt_3 solution and silica gel removed by filtration through a small glass wool plug in a Pasteur pipet. The filtrate was extracted with ether (5 × 2 mL), briefly evaporated at a water pump to remove excess ether, and made up to about 1 mL with aqueous 1% NEt_3. The dimethoxytrityl oligomer so produced was purified by reverse-phase HPLC using gradient I (retention time, 8.7 min). Usually ten aliquots of 100 μL each were injected onto the column. The fractions that contained product were pooled and evaporated to an oil at a water pump. Most of the TEAA was removed with a high-vacuum pump and repeated coevaporations with methanol. During these evaporations some detritylation occurred. The dimethoxytrityl groups were then completely 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 1 mL of water, and the solution was extracted with ether (5 × 2 mL). Excess ether was removed by a brief evaporation of the aqueous phase and the product made up to a volume of 1 mL. Final purification, by injection of ten aliquots of 100 μL each, was by reverse-phase HPLC using gradient II. Fractions that contained product were pooled and evaporated to dryness. Excess TEAB was removed by coevaporation from methanol. The purity of the oligonucleotides was checked by HPLC using gradients III, IV, and V. The purified products were dissolved in 1 mL of water and stored frozen at −20 °C. Usually between 1.5 and 3 μmol of pure octanucleotides was obtained. This represents a yield of between 15 and 30% based on the first cytidine residue attached to the silica gel.

5′-Phosphorylation of Oligonucleotides. The appropriate oligonucleotide (about 2 A_{260} units) dissolved in a 200-μL volume containing 50 mM glycine, pH 9.2, 10 mM DTT, 5 mM $MgCl_2$, and 1 mM ATP was phosphorylated with polynucleotide kinase (25 units) at 37 °C. The reaction was monitored by HPLC (gradient II) and was usually complete in 90 min. The 5′-phosphorylated oligomers were then isolated by preparative HPLC (gradient II).

Digestion of Oligonucleotides with Nuclease P1 and Alkaline Phosphatase. The appropriate oligomer (about 1 A_{260} unit) was dissolved in 200 μL of 25 mM Tris-HCl, pH 7, and digested with nuclease P1 (20 μg) for 2 h at 37 °C. Digestion was complete after this time and an aliquot was analyzed by HPLC (gradient VI). To the remaining solution were added $MgCl_2$ (to a final concentration of 10 mM) and alkaline phosphatase (10 μg). After a further 2-h incubation at room temperature the mixture was again analyzed by HPLC (gradient VII).

Desulfurization of Phosphorothioate-Containing Oligonucleotides. About 0.5 A_{260} unit of the phosphorothioate-containing oligomer dissolved in H_2O (25 μL) was reacted with iodine (0.5 mg) dissolved in pyridine (75 μL). The reaction was carried out at room temperature for 45 min. Water (1 mL) was then added and the iodine extracted with ether (5 × 2 mL). The aqueous phase was then evaporated to dryness, the residue redissolved in 100 μL of H_2O, and the product of the reaction purified by HPLC (gradient II).

Digestion of Oligonucleotides with EcoRI. Oligonucleotide (approximately 1 A_{260} unit) dissolved in a 200-μL volume containing 10 mM Tris-HCl, pH 7.6, 80 mM NaCl, and 20 mM $MgCl_2$ was digested with *Eco*RI (between 2.75 and 13.75 μg). The reaction mixtures were incubated at 16 °C for times of up to 24 h. Aliquots were analyzed by HPLC, and in the cases where cleavage took place the products were purified by HPLC (gradient II).

Results

The octamers were synthesized by the phosphite method on a solid support employing deoxynucleoside 3′-*O*-(methoxy-

morpholinophosphites) (Matteuci & Caruthers, 1981; McBride & Caruthers, 1983; Dörper & Winnacker, 1983) as the building units and Fractosil 200 (a silica-based material) as the solid phase, contained in a glass syringe (Tanaka & Letsinger, 1982). Some of the difficulties encountered in the synthesis of the all-phosphate-containing octamer d(GGAATTCC) by this method with respect to coupling times and removal of dimethoxytrityl groups will be discussed under Discussion.

To obtain a mixture of the R_P and S_P diastereomers of the phosphorothioate-containing octamer d(GGsAATTCC), the iodine–water oxidation step of the phosphite group between dA and dG was replaced by one consisting of the addition of elemental sulfur. Routinely, a suspension of sulfur in pyridine and a 2-h reaction time were used. After reaction the insoluble sulfur was removed by flushing with a CS_2–pyridine (1:1) solution. Addition of sulfur in a homogeneous solution in this solvent was tried as an alternative and gave comparable results. The addition of sulfur instead of the oxidation did not reduce the yield of the subsequent coupling step, which was ≥95% as determined spectroscopically by the liberation of the dimethoxytrityl group.

In order to prepare chirally pure oligomers, we have utilized the addition of a presynthesized chirally pure phosphorothioate dimer to the growing oligonucleotide chain. The phosphorothioate-containing d[Gp(S)A] dimer was prepared by condensing N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine 3′-(methoxymorpholinophosphite) with N^6-benzoyl-3′-*O*-[(*p*-chlorophenoxy)acetyl]-2′-deoxyadenosine using tetrazole as the activating agent. Subsequent addition of elemental sulfur yielded the fully protected phosphorothioate dimer, and a brief treatment with ammonia then removed the (*p*-chlorophenoxy)acetyl-protecting group. Purification and diastereomer separation were simultaneously achieved by silica gel chromatography. It is important to use silica gel 60 H for this separation and also the solvent mixture given under Materials and Methods. Other silica gel types and solvent systems were much less effective in diastereomer resolution. The fast and slow fractions of the required dimer product appeared pure by TLC after silica gel chromatography. Additionally, both fractions appeared pure by ^{31}P NMR spectroscopy (fast, δ 69.67; slow, δ 69.86). ^{31}P NMR spectroscopy of a 3:1 slow:fast mixture confirmed that this difference in chemical shifts was real and that the fast isomer resonates at higher field. The absolute configuration at phosphorus of the two fractions was established by the complete deblocking of a small sample of a 3:1 slow:fast mixture. Removal of the methyl groups with thiophenol occurs with C–O bond cleavage and so does not change the configuration at phosphorus (Daub & van Tamelen, 1977). ^{31}P NMR spectroscopy of the resulting mixture after this deblocking revealed two peaks in a 3:1 ratio at δ 55.89 and 54.87, respectively. Since it is known that the S_P diastereomer of dinucleoside phosphorothioates resonates at higher field than the R_P diastereomer (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982), this establishes that the fast fraction contained the isomer with the S_P configuration and the slow fraction the one with the R_P configuration. Confirmation of this result comes from reverse-phase HPLC of the deblocked mixture in which the major peak elutes before the minor. Again the R_P diastereomer of dinucleoside phosphorothioates is known to elute before the S_P in reverse-phase HPLC systems (Romaniuk & Eckstein, 1982; Bartlett & Eckstein, 1982). Finally, the major product was susceptible to digestion by snake venom phosphodiesterase but not by nuclease P1 whereas

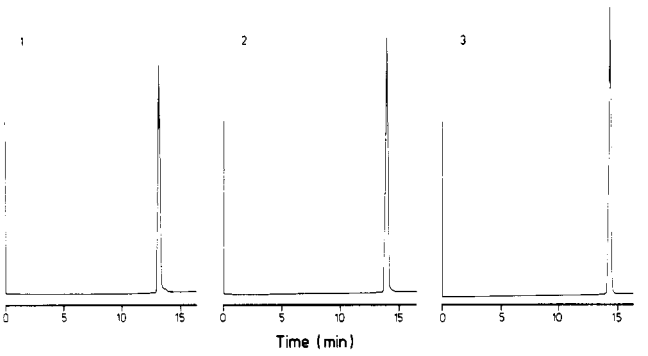

FIGURE 1: Reverse-phase HPLC analysis of octamers: (1) d(GGAATTCC), (2) (R_P)-d(GGsAATTCC), and (3) (S_P)-d(GGsAATTCC); gradient III was used.

the opposite enzyme selectivity was seen for the minor product. Again this means that the major peak has the R_P configuration and the minor the S_P (Burgers & Eckstein, 1978b; Potter et al., 1983). All these tests indicate that the slow triester has the R_P and the fast the S_P configuration. After purification the R_P and S_P diastereomers of the methyl esters of d[Gp(S)A] were separately treated with methoxymorpholinochlorophosphite, resulting in a dinucleoside phosphorothioate containing a methoxymorpholinophosphite moiety at the 3′-hydroxyl group. After further purification by silica gel chromatography these methoxymorpholinophosphite dimers can then be attached to the growing nucleotide chain in the usual fashion. Subsequent to the coupling of this dinucleoside phosphorothioate, one more coupling with 2′-deoxyguanosine 3′-*O*-(methoxymorpholinophosphite) and an oxidation step have to be performed to complete the synthesis of the octamer.

After completion of the solid-phase synthesis the methyl-protecting groups were removed from the phosphate and phosphorothioate triesters with thiophenolate. The base-protecting groups were then removed, and the oligonucleotide was simultaneously cleaved from the silica gel by ammonia treatment. The oligonucleotide, containing a dimethoxytrityl group at the 5′-terminus, was then purified by reverse-phase HPLC. All the truncated sequences resulting from incomplete coupling yields followed by capping with acetic anhydride do not contain a highly hydrophobic dimethoxytrityl group and therefore elute much earlier than the desired product on reverse-phase HPLC. The purified dimethoxytrityl oligomer was then treated with acetic acid to remove the dimethoxytrityl-protecting group and finally purified further by HPLC. For this final purification TEAB was used as the buffer salt in conjunction with an acetonitrile gradient. All the components used in this step are volatile and are easily removed by evaporation, eliminating the need for a final desalting step.

The purified oligonucleotides were 5′-phosphorylated by using polynucleotide kinase with ATP as the phosphoryl donor. This reaction was monitored by reverse-phase HPLC, as the phosphorylated products elute before the starting octamers. The same system was used for the purification of the 5′-phosphorylated oligonucleotides.

The purity of the oligomers produced has been checked by reverse-phase HPLC using either KH_2PO_4, TEAA, or tetrabutylammonium phosphate as buffer with an acetonitrile gradient. In all these systems d(GGAATTCC) as well as (S_P)- and (R_P)-d(GGsAATTCC) appeared ≥95% pure (Figure 1). The all-oxygen-containing compound always eluted earlier than the phosphorothioate-containing oligomers with base line separation being achieved. No separation was observed between the S_P and R_P isomers of d(GGsAATTCC), which when coinjected eluted as a single symmetrical peak. The 5′-

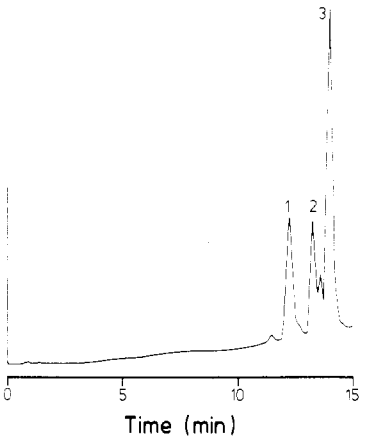

FIGURE 2: Reverse-phase HPLC analysis of 5′-phosphorylated octamers. The solution was prepared by mixing solutions containing the individual isomers: (1) d(pGGAATTCC), (2) (S_P)-d(pGGsAATTCC), and (3) (R_P)-d(pGGsAATTCC); gradient V was used. The small peak between peaks 2 and 3 is due to a contaminant in (R_P)-d(GGsAATTCC).

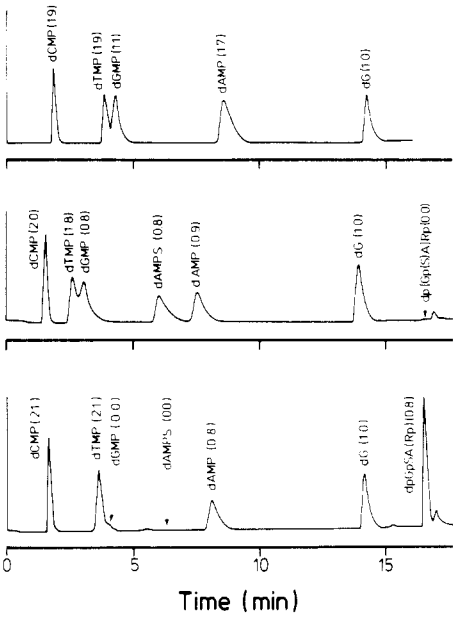

FIGURE 3: HPLC analysis of nuclease P1 digest of octamers: digests of d(GGAATTCC) (top), (R_P)-d(GGsAATTCC) (middle), and (S_P)-d(GGsAATTCC) (bottom); gradient VI was used. The numbers in parentheses refer to the equivalent amounts of each of the nucleotides by integration.

phosphorylated oligomers were also analyzed by reverse-phase HPLC using the KH_2PO_4 and TEAA buffer systems. d(pGGAATTCC) and (S_P)-d(pGGsAATTCC) appeared ≥95% pure. As for the nonphosphorylated octamers, the all-oxygen-containing nucleotide eluted before those containing sulfur. Remarkably, the two phosphorylated phosphorothioate oligonucleotide diastereomers were base line resolved, with the S_P isomer eluting before the R_P (Figure 2). The individual 5′-phosphorylated phosphorothioate diastereomers showed negligible contamination with the other isomer, indicating that the original unphosphorylated oligomers must also have been of a very high diastereomeric purity.

The oligonucleotides were further characterized by nuclease P1 digestion followed by analysis of the products by HPLC. Nuclease P1 cleaves nucleotides giving nucleoside 5′-phosphates and so d(GGAATTCC) would be expected to yield dG, dGMP, dAMP, dTMP, and dCMP in a ratio of 0.5, 0.5, 1.0, 1.0, and 1.0, respectively. This is indeed the case as is shown (Figure 3). Further treatment of this mixture with alkaline phosphatase gave dG, dA, dT, and dC in the expected equimolar ratios. Phosphorothioates having the S_P configuration are digested by nuclease P1 (Potter et al., 1983; S. Spitzer and F. Eckstein, unpublished results), and so, for example, (S_P)-d[Gp(S)A] would yield dG and dAMPS. Phosphorothioates having the R_P configuration are not cleaved by nuclease P1 (Potter et al., 1983). Thus (S_P)-d(GGsAATTCC) would be expected to give dG (0.5), dGMP (0.5), dAMPS (0.5), dAMP (0.5), dTMP (1.0), and dCMP (1.0) after nuclease P1 treatment (the figures in parentheses refer to the equivalents expected) as is indeed found (Figure 3). After the addition of alkaline phosphatase the 5′-monophosphates were converted to deoxynucleosides, giving the ratios expected. dAMPS is inert to alkaline phosphatase and so remains unchanged. Treatment of (R_P)-d(GGsAATTCC) with nuclease P1 gave dG (0.5), (R_P)-d[pGp(S)A] (0.5), dAMP (0.5), dTMP (1.0), and dCMP (1.0) as expected and shown (Figure 3). The identification of d[pGp(S)A] in Figure 3 is tentative as we do not possess this compound as a standard. After alkaline phosphatase treatment, however, the (R_P)-d[Gp(S)A] formed can be conclusively identified by comparison with standard material. Additionally, the other dephosphorylated nucleosides were produced in the expected ratios. This analysis not only establishes that the base composition of the synthesized octamers is correct but also proves that the phosphorothioate oligomer of the S_P configuration is derived from the S_P-protected d[Gp(S)A] dimer and that the R_P oligomer is derived from the R_P dimer. This analysis therefore confirms that very little, if any, epimerization at phosphorus occurs during the entire synthesis, deblocking, and purification of the phosphorothioate oligomers.

Treatment of the phosphorothioate-containing oligomers with iodine in pyridine resulted in desulfurization and formation of the normal all-phosphate-containing nucleotide. This reaction was monitored by HPLC and appeared to be both quantitative and free from side reactions. All the d(GGsAATTCC) was converted to a product identical with d(GGAATTCC) by use of several HPLC systems. Further proof of the integrity of the d(GGAATTCC) so produced was that it was completely digested by *Eco*RI, yielding the expected products d(GG) and d(pAATTCC). A similar desulfurization of a [pAp(S)U] copolymer with iodine has previously been reported (Burgers & Eckstein, 1979).

The midpoint of thermal transition of d(GGAATTCC) and the two phosphorothioate oligomers all lie between 23 and 25 °C. Thus, within the experimental error of the method, the various oligomers have similar, if not identical, thermal stabilities.

The negative ion FAB mass spectrum of the "fast" isomer of d(GGsAATTCC) together with the expected breakdown shown in the structural formula is given in Figure 4. The data are summarized in Table I. The background of the spectrum is higher than those given in Grotjahn et al. (1982) for all-phosphate-containing oligomers. The main reason for this background seems to be the smaller homogeneity as well as the incomplete exchange of Na^+ ions for triethylammonium ions, as can be seen from the molecular and sequence ions that are accompanied throughout by the corresponding Na^+-containing masses. The deprotonated molecular ion appears at 2423 d. The 5′-phosphate sequence ions could be registered up to the fifth nucleotide and and 3′-phosphate sequences up to the third. More sequence ions could not be assigned since they are buried in the background. The sequence ions are usually accompanied by relatively intense −18 d (loss of H_2O) and Na^+-containing 22 d (−H, +Na^+) ions.

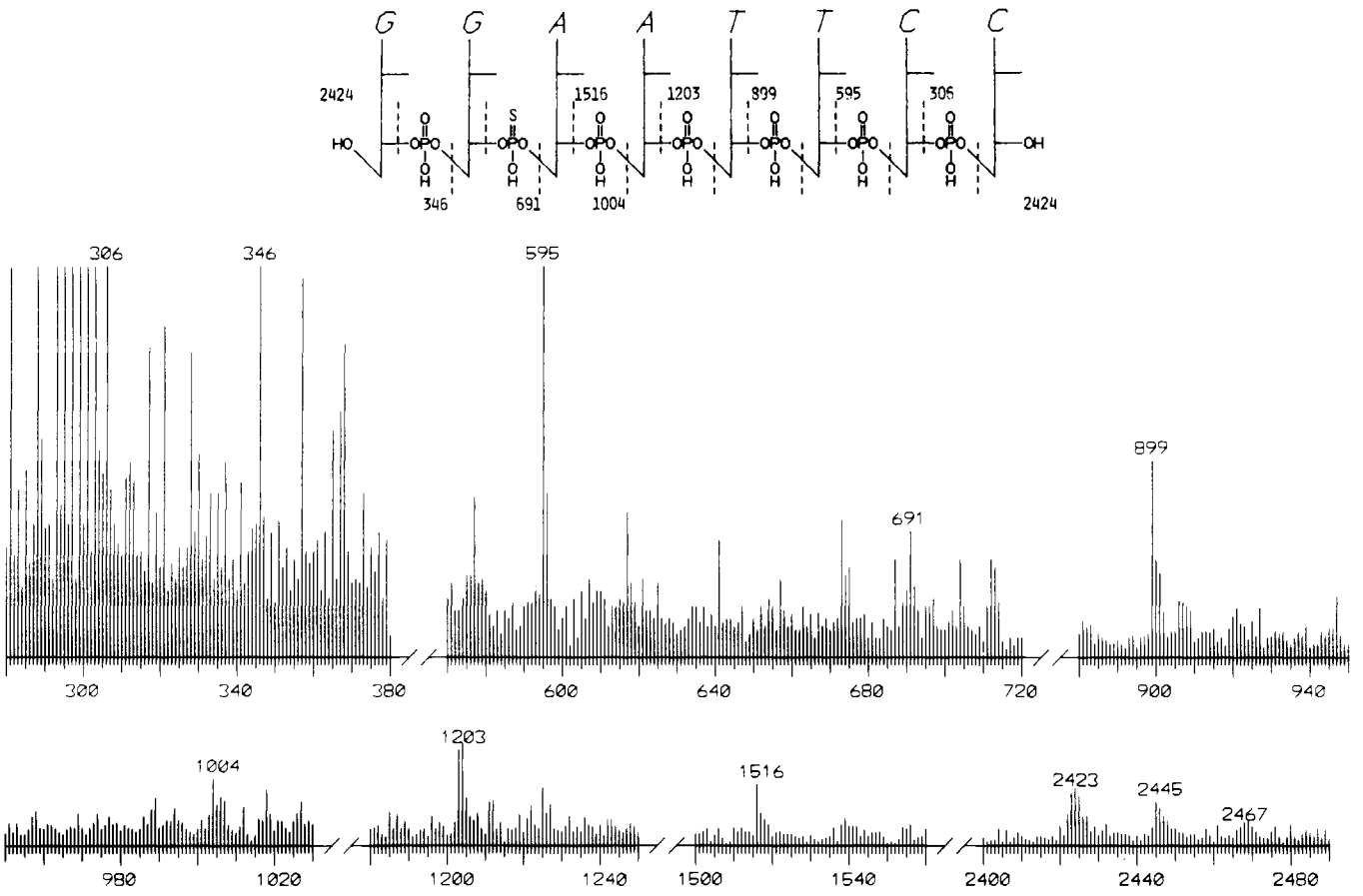

FIGURE 4: Negative ion FAB mass spectrum of (S_P)-d(GGsAATTCC).

Table I: Masses of Sequence Ions Observed in FAB Mass Spectrometric Analysis of d(GGAATTCC) and d(GGsAATTCC)

	d(GGAATTCC)[a]		d(GGsAATTCC)[b]	
	5′-P	3′-P	5′-P	3′-P
(1) sequence ion	306	346	306, 288 ($-H_2O$), 328 (−H + Na)	346, 328 ($-H_2O$), 368 (−H + Na)
(2) sequence ion	595	675	595, 577 ($-H_2O$), 617 (−H + Na)	691, 673 ($-H_2O$), 713 (−H + Na)
(3) sequence ion	899	988	899, 881 ($-H_2O$), 921 (−H + Na)	1004, 986 ($-H_2O$), 1026 (−H + Na)
(4) sequence ion	1203	1301	1203, 1185 ($-H_2O$), 1225 (−H + Na)	
(5) sequence ion	1516	1605	1516, 1538 (−H + Na)	
(6) sequence ion	1829	1909		
(7) sequence ion	2158	2198		
(8) deprotonated molecular ion	2407		2423, 2445 (−H + Na), 2467 (−2H + 2Na)	

[a] Data taken from a spectrum not shown. [b] Data from the spectrum shown in Figure 4.

The ^{31}P NMR spectrum of d(GGAATTCC) taken at 10 °C shows a group of resonances between δ −3.9 and −4.5 (Figure 5). The spectrum of (S_P)-d(GGsAATTCC) shows in addition a signal of intensity 1.0 at δ 50.76 representing the resonance of the phosphorothioate group. Only one signal is observed, indicating high diastereomeric purity. The spectrum of the mixture of diastereomers of d(GGsAATTCC) shows two resonances at δ 51.16 and 50.74 in an approximate ratio of 1:1, confirming what was found from the nuclease P1 digest of the mixture of diastereomers, namely, that sulfur addition to the oligonucleotide proceeds without any detectable stereoselectivity.

Both d(GGAATTCC) and d(pGGAATTCC) were digested by *Eco*RI to give only two products as monitored by HPLC. Collection of these products and analysis using the nuclease P1 and nuclease P1–alkaline phosphatase treatment showed that these products were d(GG) and d(pAATTCC) in the case of d(GGAATTCC) and d(pGG) and d(pAATTCC) in the case of the phosphorylated derivative. The 5′-phosphorylated octamer was hydrolyzed faster than d(GGAATTCC). Thus, under our standard conditions as described under Materials and Methods using approximately 13.75 μg of enzyme, the cleavage of the phosphorylated octamer had proceeded to about 50% in 1 h whereas cleavage of the unphosphorylated octamer had only occurred to about 6%. Treatment of the R_P isomers of both d(GGsAATTCC) and d(pGGsAATTCC) with *Eco*RI also resulted in cleavage at a single point with two products being formed (Figure 6). Collection and analysis of the products showed that they were d(GG) and d[p(S)-AATTCC] in the case of the unphosphorylated oligomer and d(pGG) and d[p(S)AATTCC] for the phosphorylated species. Again the 5′-phosphorylated octamer was cleaved at a faster rate than the unphosphorylated octamer. With the same amount of enzyme as above the reaction with the former had

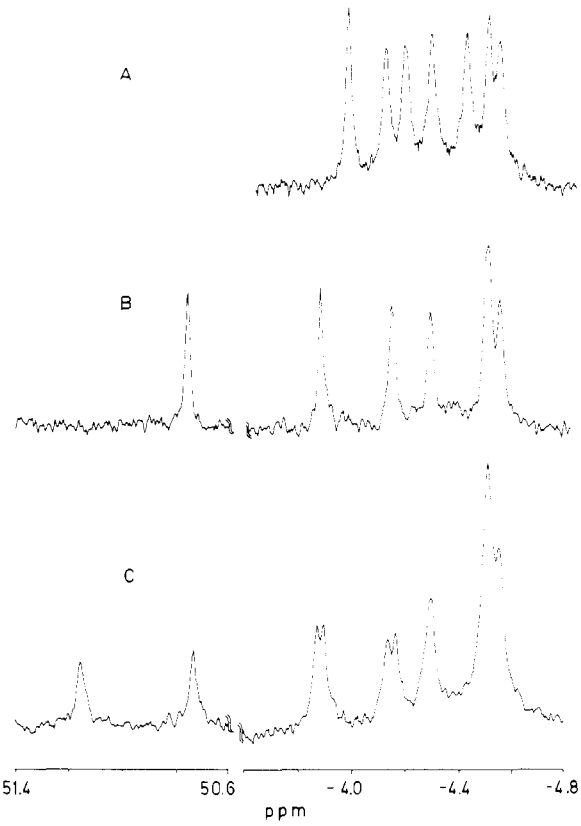

FIGURE 5: ^{31}P NMR spectra of octamers: (A) d(GGAATTCC); (B) (S_P)-d(GGsAATTCC); (C) mixture of (R_P)- and (S_P)-d(GGsAATTCC). Spectra were recorded at 10 °C. Parameters were as follows: offset, 1350 (A) and 3800 Hz (B and C); sweep width, 800 (A) and 6024 Hz (B and C); pulse width, 5.5 (A) and 4.0 μs (B and C); 16K (A) and 32K (B and C); acquisition time, 10.24 (A) and 2.7 s (B and C); line broadening, 0.4 (A) and 0.5 Hz (B and C); number of transients, 916 (A), 1530 (B), and 1000 (C). Chemical shifts are relative to trimethyl phosphate.

reached 75% completion after 20-h incubation, whereas reaction with the latter had only occurred to 10% completion. Because of the scarcity of material no detailed kinetic studies could be undertaken with any of the octamers so that all the values given here obtained by measurement of one or two time points are only approximate.

The S_P isomers of d(GGsAATTCC) and d(pGGsAATTCC) were not cleaved even after incubation for 30 h.

Discussion

The observation that at least certain restriction endonucleases including *Eco*RI can cleave phosphorothioate internucleotidic linkages albeit more slowly than phosphate linkages (Vosberg & Eckstein, 1982; B. V. L. Potter, H. P. Vosberg, and F. Eckstein, unpublished results) led us to attempt the synthesis of an oligonucleotide containing the recognition sequence for such an enzyme and possessing a phosphorothioate group at the site of cleavage. It was envisaged that such a compound would be a substrate and should be suitable for the determination of the stereochemical course of the enzyme reaction, employing methods that have been applied to a large range of phosphoryl and nucleotidyl transferring enzymes [see review for Eckstein (1983a,b)]. The most suitable enzyme for study seemed to be the enzyme *Eco*RI since in the class of restriction endonucleases it is the most thoroughly investigated, and thus any information on the stereochemistry of the reaction would be a most useful additional detail for the description of a mechanism (Modrich, 1982). Moreover, it is an enzyme most easily obtainable in sufficient quantities due to the existence of an overproducing strain. This enzyme is known to cleave the octanucleotides d(pTGAATTCC) (Greene et al., 1975) and d(pGGAATTCC) (Goppelt et al., 1980). The latter has a higher thermal stability than the former, and it was thus decided to synthesize the octanucleotide d(GGsAATTCC) that contains the recognition sequence for *Eco*RI with a phosphorothioate between dG and dA, the position of cleavage.

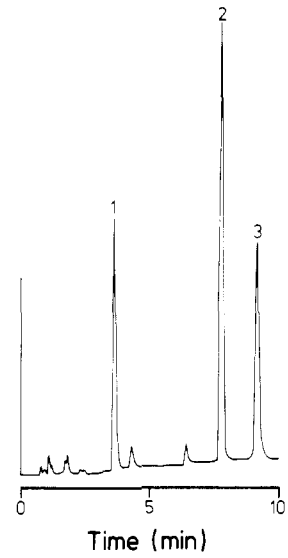

FIGURE 6: HPLC analysis of a partial *Eco*RI digestion of (R_P)-d(pGGsAATTCC): (1) d(GG), (2) d[p(S)AATTCC], and (3) (R_P)-d(GGsAATTCC); gradient II was used; incubation conditions as described under Materials and Methods.

Modern methods of oligonucleotide synthesis are based on either the phosphotriester (Marugg et al., 1983; Gait et al., 1982a,b; Ito et al., 1982; Köster et al., 1983) or the phosphite methodologies (Matteuci & Caruthers, 1981). In the latter a phosphite internucleotidic linkage is formed first, which is oxidized in a second step with iodine–water to a phosphate linkage. It has been shown in solution that such dinucleoside phosphite triesters can be converted to the corresponding dinucleoside phosphorothioate triesters by addition of sulfur instead of oxidation with iodine–water (Burgers & Eckstein, 1978a; Marlier & Benkovic, 1980). Additionally, deblocking of methyl phosphotriesters by thiophenol proceeds with C–O bond cleavage (Daub & van Tamelen, 1977), eliminating the possibility of epimerization at phosphorus during triester to diester conversion. Thus, the phosphite methodology seemed very attractive for the synthesis of the modified octamer as very little modification of the existing methodology was needed.

The phosphite approach based on morpholinomethoxyphosphine was chosen as the starting materials are easy to prepare and purify and, in addition, are very stable when dissolved in the usual solvents used for oligonucleotide synthesis (McBride & Caruthers, 1983; Dörper & Winnacker, 1983). Furthermore, solid-phase synthetic methods, as opposed to those conducted in solution, greatly simplify both the synthesis and the subsequent purification of the oligomers prepared (Matteuci & Caruthers, 1981). With these considerations in mind we chose to prepare the desired phosphorothioate-containing octanucleotide and also, as a control, the corresponding all-phosphate-containing octamer by a solid-phase method using nucleoside methoxymorpholinophosphites as building blocks.

During the synthesis of d(GGAATTCC), which has also been synthesized by a polymer-supported phosphotriester ap-

proach earlier (Oktsuka et al., 1982), we noticed that the times required to couple the incoming nucleoside methoxymorpholinophosphites to the free 5′-hydroxyl group, using tetrazole as the activating agent, were somewhat greater than those recommended for the nucleoside methoxydimethylaminophosphite method. For this latter method a 5-min reaction time suffices, whereas all couplings except the initial one using the morpholino derivative required 10 min to go to completion. The first coupling appeared to be especially slow, and 30 min was necessary in order to obtain ≥95% coupling yields. A similarly slow initial coupling step was found when nucleoside methoxydiisopropylaminophosphines and silica gel were used as the solid support (Adams et al., 1983). These authors suggested that steric hindrance was the cause of this slow reactivity and showed that changing the support to controlled pore glass appeared to overcome this problem. Recently, McBride & Caruthers (1983) have demonstrated that nucleoside methoxymorpholinophosphines are less reactive than the corresponding dimethylamino derivatives. Fröhler & Matteucci (1983) showed that the use of (*p*-nitrophenyl)-tetrazole, instead of tetrazole, as the activating agent greatly speeded up the reaction rates with nucleoside methoxymorpholinophosphites. Presumably, incorporation of these two modifications will drastically reduce the total synthesis time. Additionally, we have found that the use of $ZnBr_2$ as the detritylating agent is unsatisfactory. This Lewis acid was suggested as a replacement for protic acids as it does not cause depurination of N^6-benzoyldeoxyadenosine residues under conditions where it removed dimethoxytrityl groups (Matteucci & Caruthers, 1981). However, we have observed detritylation with this reagent (used as a saturated solution in CH_3NO_2–CH_3OH, 95:5) to be slow and incomplete, especially for the sequence DMTdCC. The use of 10% trichloroacetic acid dissolved in dichloroethane for 2 min caused complete detritylation without significant depurination (Gait et al., 1982). Providing that the coupling times mentioned above and under Materials and Methods are followed and trichloroacetic acid is used to remove dimethoxytrityl groups, each coupling step proceeds ≥95% yield, as judged by dimethoxytrityl cation release.

Two alternative approaches were considered for the synthesis of d(GGsAATTCC). First, addition of sulfur instead of oxygen to the growing oligonucleotide chain at the stage when the crucial dG–dA phosphite linkage had been synthesized, and second, addition of a preformed and suitable protected stereochemically pure d[Gp(S)A] derivative as a block. The first approach is attractive because of its simplicity but has the disadvantage that the stereochemistry of addition of sulfur cannot be controlled. Since it originally seemed highly unlikely that the mixture of diastereomers produced could be separated by any chromatographic method, this method was initially used to produce such an octamer quickly and provide material to study various properties, particularly those important for separation and characterization. The second method offered the advantage that if $DMTdG^{ib}p(S,OMe)dA^{bz}_{OH}$ could be separated into its diastereomers and could be added as a block, an octamer containing a phosphorothioate of known configuration would be obtainable, a prerequisite for the envisaged stereochemical studies. Both methods have in common that after introduction of the phosphorothioate triester one additional coupling has to be performed to introduce the terminal dG residue. To ascertain that the iodine–water oxidation step necessary for the formation of this last internucleotidic linkage did not cause desulfurization of the phosphorothioate triester, studies with $DMTdG^{ib}p(OMe,S)dA^{bz}_{OH}$ as a model were undertaken. They showed that even after treatment of up to 1 h with a 1% iodine solution in THF–lutidine–H_2O no such desulfurization occurred. This inertness of the phosphorothioate triesters contrasts with the facile desulfurization of phosphorothioate diesters by iodine dissolved in pyridine.

As expected, the first route produced an octamer comprising a 50:50 mixture of the R_P and S_P isomers of d(GGsAATTCC). This was confirmed by both ^{31}P NMR spectroscopy and digestion with nuclease P1. Supportive evidence for the structure of this phosphorothioate octamer was provided by the desulfurization reaction with iodine. This reaction proceeded in a remarkably clean manner to give the unmodified oligonucleotide d(GGAATTCC), as was evidenced both by HPLC and by its complete cleavage by *Eco*RI to the expected products d(GG) and d(pAATTCC). Although this reaction was not investigated in detail, it became apparent that pyridine is essential for it to proceed. Three other methods are available for the desulfurization of phosphorothioates, namely, the uses of cyanogen bromide (Sammons & Frey, 1982), *N*-bromosuccinimide (Connolly et al., 1982), and bromine (Lowe et al., 1982). With the last two methods side reactions with some of the bases, especially guanosine, occur, which contrasts with the mildness of the iodine method. However, the stereochemical course of the iodine-mediated desulfurization has yet to be determined as it has been for the three other methods.

The second route necessitated the synthesis of $DMTdG^{ib}p(OMe,S)dA^{bz}_{OH}$. One of the problems encountered in this synthesis was the proper choice of intermediary protection of the 3′-OH group of dA. The strategy required that this protecting group had to be removed without hydrolysis of the phosphotriester to allow phosphitylation of the 3′-OH group of dA. The (*p*-chlorophenoxy)acetyl group was selected since it can be removed by brief treatment with dilute ammonia (van Boom et al., 1971), conditions under which the phosphorothioate triester was stable. The two diastereomers of $DMTdG^{ib}p(OMe,S)dA^{bz}_{OH}$ could be separated by silica gel chromatography and their absolute configuration determined after complete deblocking by digestion with nuclease P1, ^{31}P NMR spectroscopy, and HPLC. After reaction with morpholinomethoxychlorophosphine and purification of the products, these two diastereomers could be used as blocks in the octamer syntheses. The final octamers were shown to be diastereomerically pure by ^{31}P NMR spectroscopy and nuclease P1 digestion. Additionally, the nuclease P1 digestion confirmed the expected nucleotide composition. Also, both the R_P and the S_P diastereomers of d(GGsAATTCC) were desulfurized with iodine to produce d(GGAATTCC), a further proof of the structure.

The mixture of diastereomers produced by the first method and the separate diastereomers produced by the second could both be easily phosphorylated at the 5′-OH groups by polynucleotide kinase. Most remarkable was the finding that although the unphosphorylated diastereomers could not be separated by HPLC, the 5′-phosphorylated species were separable. This is of practical consequence as the first method of synthesis is much easier than the second, allowing the rapid preparation of large amounts of material. Phosphorylation then becomes the handle allowing separation of the mixture of diastereomers produced by this procedure by HPLC.

We felt that a completely independent check ought to be made on the composition and sequence of the phosphorothioate octamer. Normally, a wandering spot sequence analysis should be performed on such an oligonucleotide (Brownlee & Sanger 1969; Jay et al., 1974). However, the stereoselectivity of most nucleases for one or the other diastereomer of a dinucleoside

phosphorothioate poses problems for the general application of this method to the analysis of phosphorothioate-containing oligomers. It was therefore decided to try FAB mass spectrometry for the analysis of the phosphorothioate octamers as this method has been shown to be capable of analyzing oligonucleotides up to a chain length of ten (Grotjahn et al., 1982). The presence of Na^+, which we were unable to remove by various chromatographic methods, represented one of the main difficulties in this analysis. Nevertheless, the mass spectrum for the S_P isomer (a similar one has been obtained for the R_P isomer) (Figure 4) shows as detailed in Table I that the fragmentation pattern is fully compatible with the structure of the octamer being d(GGsAATTCC). Of particular importance is overlap for the fragmentation from the 3′ and 5′ ends. Fragments from the 3′ end yielding nucleotide 5′-phosphates can be identified up to the fifth nucleotide and those from the 5′ end yielding nucleotide 3′-phosphates up to the third nucleotide including the crucial d[Gp(S)A] part. Thus, this analysis shows that FAB mass spectrometry can successfully be applied to the analysis of modified oligonucleotides. For many modified oligonucleotides this might be the method of choice, particularly for those where the phosphate group has been altered and rendered resistant to nucleases.

A very important characteristic of these octamers is their thermal stability, especially as *Eco*RI requires a double-stranded structure as substrate. This is significant since a decreased thermal stability has been documented for the phosphorothioate analogues of the alternating polynucleotides poly[d(G-C)] and poly[d(A-T)] (Eckstein & Jovin, 1983; Jovin et al., 1983). In these polymers the thermal stability is lowered to the greatest extent when the pyrimidine nucleoside 5′-phosphate is substituted by a phosphorothioate. For poly[d[pGp(S)C]] a decrease in T_m of 8 °C was observed whereas for poly[d[pAp(S)T]] the T_m was lowered by 15 °C. For the polymers containing a purine nucleoside 5′-phosphorothioate such as poly[d[pCp(S)G]] and poly[d[pTp(S)A]] the T_m values were lowered 2 and 5 °C, respectively. However, contrary to this it was found that both (R_P)- and (S_P)-d(GGsAATTCC) as well as the mixture of diastereomers had T_m values of between 23 and 25 °C, similar to that of d(GGAATTCC).

As a further characterization the ^{31}P NMR spectra of all the octamers were recorded. At 10 °C, conditions where these octamers exist as double helices in the buffer system used, the spectrum of d(GGAATTCC) clearly shows seven resonances of the same intensity whereas at 40 °C (not shown), much less resolution was observed. A similar spectrum recorded at 30 °C has been reported by Patel & Canuel (1979). At present we are unable to assign any of these signals to a particular phosphate group in the oligonucleotide sequence. On the basis of what has been observed for the phosphorothioate analogues of poly[d(A-T)] and poly[d(G-C)] (Eckstein & Jovin, 1983; Jovin et al., 1983), it was expected that the spectra of the phosphorothioate and the all-phosphate-containing octamers should be very similar with the exception that the signal arising from the phosphorothioate of d[Gp(S)A] would be missing from this part of the spectrum since the phosphorothioates resonate at much lower field. By this analysis at least one of the phosphate resonances of the unmodified octamer should have been assignable. However, the spectrum of (S_P)-(dGGsAATTCC) recorded at 10 °C does not fit such a pattern. Some resonances seem to be unaltered in the phosphorothioate such as those at δ −4.54, −4.50, −4.34, and −4.15, but more than one have either disappeared or shifted relative to the spectrum of the unmodified octamer. Thus, the resonances of the phosphorothioate octamer at δ −4.54 and −4.50 integrate to 3 rather than 2 equiv. This must be an indication of the changes in conformation caused by the mixture of diastereomers where fine structure is seen in two of the phosphate resonances, indicating differential perturbation presumably of the neighboring phosphates by the two isomers.

As expected, d(pGGAATTCC) was cleaved by *Eco*RI, yielding the appropriate dimer and hexamer. It was also found that the unphosphorylated octamer was a substrate for the enzyme. However, this was cleaved approximately 8 times more slowly than the phosphorylated species. This observation is similar to that of Dwyer-Hallquist et al. (1982), who found in a more detailed study that the enzyme *Hpa*I cleaves d(pGGTTAACC) about 30 times faster than the unphosphorylated octamer. Of the two diastereomers of d(GGsAATTCC) and d(pGGsAATTCC), only the R_P isomers were hydrolyzed. Also, in this case the phosphorylated octamer was cleaved faster by a factor of about 7. These results probably indicate, as suggested by Dwyer-Hallquist et al. (1982), that the 5′-terminal phosphate of this octamer is also part of the recognition sequence of the *Eco*RI enzyme. The observed stereospecificity is in line with what has been found for the hydrolysis by restriction enzymes of enzymatically synthesized DNA-containing phosphorothioate groups in one strand only (Vosberg & Eckstein, 1982; B. V. L. Potter, H. P. Vosberg, and F. Eckstein, unpublished results). In such DNA the phosphorothioate groups are of the R_P configuration, and they were indeed cleaved by the restriction enzymes investigated although at a slower rate than unmodified DNA. Since enzymatic formation of a phosphorothioate internucleotidic linkage by DNA polymerases always produces the R_P configuration (Eckstein, 1983a,b), the stereospecificity of restriction enzymes can only be determined by the chemical synthesis of the phosphorothioate linkage as demonstrated in this publication. The limited kinetic study we were able to carry out indicates that the phosphorylated octamers as well as the unphosphorylated (R_P)-phosphorothioate octamers are cleaved approximately 15 times more slowly than the corresponding all-phosphate-containing octamers. We are at present using (R_P)-d(pGGsAATTCC) to evaluate the stereochemical course of the *Eco*RI catalyzed reaction.

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Registry No. *Eco*RI, 80498-17-5; d(GGAATTCC), 70755-49-6; (R_P)-d(GGsAATTCC), 90584-24-0; (S_P)-d(GGsAATTCC), 90639-14-8; d(pGGAATTCC), 71065-77-5; (R_P)-d(pGGAATTCC), 90584-25-1; (S_P)-d(pGGAATTCC), 90639-15-9; (R_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{mmp}$, 90584-28-4; (S_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{mmp}$, 90639-17-1; (R_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{OH}$, 90584-27-3; (S_P)-DMTdG^{ib}p(S,OCH$_3$)dA$^{bz}_{OH}$, 90639-16-0; (*p*-chlorophenoxy)acetic anhydride, 34359-78-9; (*p*-chlorophenoxy)acetic acid, 122-88-3; N^6-benzoyl-3′-*O*-[(*p*-chlorophenoxy)acetyl]-2′-deoxyadenosine, 90584-26-2; N^6-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxyadenosine, 64325-78-6; N^2-isobutyryl-5′-*O*-(dimethoxytrityl)-2′-deoxyguanosine 3′-*O*-(morpholinomethoxyphosphine), 86030-51-5; morpholinomethoxychlorophosphine, 86030-42-4; N^4-anisoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine 3′-*O*-(morpholinomethoxyphosphine), 90584-29-5.

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