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Alkyl phosphotriester modified oligodeoxyribonucleotides. V. Synthesis and absolute configuration of R_p and S_p diastereomers of an ethyl phosphotriester (Et) modified *Eco*RI recognition sequence, d[GGAA(Et)TTCC]. A synthetic approach to regio- and stereospecific ethylation-interference studies

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ABSTRACT

Protected deoxynucleoside 3'-O-ethyl-N,N-diisopropylphosphoramidite reagents were prepared for use in the automated synthesis of ethyl phosphotriester (Et) modified oligonucleotides. The title diastereomers were separated by reversed-phase HPLC, and chirality at phosphorus was assigned by an improved configurational correlation scheme that was verified by NMR spectroscopic studies (accompanying paper, Part VI). This generally applicable correlation scheme involved (1.) enzymatic digestions of each diastereomer to give the corresponding diastereomer of d[A(Et)T]; (2.) phosphite triester sulfurization to obtain diastereomeric O-ethyl phosphorothioates, d[A_S(Et)T], which were separated by HPLC for (3.) stereoretentive oxidation with H_2O_2 to give d[A(Et)T], and (4.) stereoretentive de-ethylation with PhSH-Et_3N to give diastereomeric phosphorothioates, d[A_ST], whose configurations at phosphorus had been assigned previously. Neither the R_p-R_p nor S_p-S_p duplex, {d[GGAA(Et)TTCC]}$_2$, was cleaved by EcoRI endonuclease under conditions that led to cleavage of both the unmodified duplex, [d(GGAATTCC)]$_2$, and the mixture of diastereomeric phosphorothioate-modified duplexes, [d(GGAA_STTCC)]$_2$. Cleavage of the latter substrates was S_p-selective.

INTRODUCTION

Investigations of the effects of alkylating agents on the structure and function of DNA are important for understanding the molecular mechanisms of carcinogenesis, mutagenesis, and cytotoxicity.[1] Such studies have generally involved the reaction of DNA with an alkylating agent, measurement of the effect(s) of interest, and then degradation of the altered DNA to identify the base- and phosphate-modified residues. Modifications of DNA by alkylating agents are also important for alkylation-interference and alkylation-protection experiments that can be used to deduce base- and phosphate-contacts with enzymes.[2] Alternative strategies for studying structural and biological effects of alkylating agents and their alkylation-interference and protection patterns are now feasible by adapting either manual or automated[3] procedures for synthesis of DNA to allow construction of specifically modified oligonucleotides, such as those bearing

O^6-methylguanine[4] and N^6-methyladenine[5] residues used to investigate the effect of base alkylation on helical structure, thermal stability, and cleavage by nucleases. Other base-modified oligonucleotides have been used to infer specific DNA contacts with restriction endonucleases,[6] repressor proteins,[7] and a monoclonal anti-native DNA autoantibody.[8]

By comparison to the variety of synthetic base-modified oligonucleotides which have been studied, investigations of backbone-modified oligonucleotides have been limited--mostly to phosphorothioate[8-10] and some alkanephosphonate[11,12] linkages. Our interests in this area led us to consider the potential utility of synthetic alkyl phosphotriester modified oligonucleotides as (1.) structural models for studying phosphate-alkylated DNA, (2.) probes for inferring phosphate-protein contacts, and (3.) stereochemical synthons for obtaining chiral phosphate and chiral phosphorothioate linkages. In addition to investigating the synthesis of these triesters, we wished to develop generally applicable chemical and spectroscopic methods for assigning the absolute configuration at phosphorus. These independent analytical methods could be used to cross-check each other and would each offer certain advantages.

The R_p and S_p configurations of several dinucleoside methyl[13-16a] and ethyl[16b] phosphotriesters have been recently assigned; however, to our knowledge, configurational assignments have not been reported for longer ethyl phosphotriester modified oligonucleotides.[16c, 17-19]

Our earlier investigations of phosphate-alkylated oligonucleotides dealt with the synthesis, characterization and stereochemistry of isopropyl phosphotriesters,[20,21] while more recent work, which has been briefly described,[22,23] included extensions to ethyl phosphotriesters. Here we report details regarding the synthesis of an ethyl phosphotriester (Et) modified octanucleotide, d[GGAA(Et)TTCC], and the application of improved chemical methods for assigning the absolute configuration at phosphorus in the HPLC-separated diastereomers of both this octanucleotide and the related dimer, d[A(Et)T], the latter of which had been previously studied in connection with backbone-alkylation of poly[d(AT)].poly[d(AT)] by 1-ethyl-1-nitrosourea.[19] A novel regio- and stereospecific approach to ethylation-interference studies is exemplified using the duplexed octamer, $\{d[GGAA(Et)TTCC]\}_2$, and *Eco*RI endonuclease. The protected deoxynucleoside 3'-*O*-ethyl-*N*,*N*-diisopropylphosphoramidites that are described herein can be used to synthesize ethyl phosphotriester modified oligonucleotides for systematic studies of DNA recognition[24] and other biologically related

processes such as phosphotriester de-ethylation in cells[17d] and sequence-specific alkylation, intercalation and binding of nucleic acids.[25]

EXPERIMENTAL

Unless specified otherwise, previously described[10,12] general procedures were followed for the automated synthesis of oligonucleotides using O-methyl, O-β-cyanoethyl, and O-ethyl phosphoramidite reagents, without treatment of the solid support with thiophenol(PhSH)-Et_3N. Unless specified otherwise, HPLC[26] gradients employed with a Waters μBondapak C_{18} column (7.8 mm x 30 cm) at 4 mL/min were as follows: gradient I = 20-30% CH_3CN (A) in 0.1 M triethylammonium acetate pH 7.0 (B) at 0.5%/min; gradient II = 10-25% A in B at 0.5%/min; gradient III = 5-40% A in B at 1.0%/min; gradient IV = 5-10% A in B at 1% min for 5 min, then 10-15% A in B at 0.25%/min for 20 min; gradient V = 1-26% A in B at 1%/min for 25 min at 3 mL/min; gradient VI = 5-20% A in B at 0.5%/min at 3 mL/min; gradient VII, A/B ratios and times: 5/95, 0; 7/93, 2; 14/86, 32 min at 3 mL/min. A poly(styrene-divinylbenzene) HPLC column (PRP-1,[27] 7 mm x 30 cm) was purchased from Hamilton, and a Zorbax ODS (C_{18}) HPLC column (21.5 mm x 25 cm) was obtained from DuPont.

Synthesis of Protected 2'-Deoxynucleoside 3'-O-ethyl-N,N-diisopropyl-phosphoramidites

O-Ethyl-N,N-diisopropylphosphoramidochloridite (386 mg) [bp 60.0-60.5 °C/0.4 Torr, δ_P = 156.3 ppm (80% H_3PO_4)], which was synthesized by the method described[28] for its O-methyl analogue, was added dropwise (2 min) under an atmosphere of argon to a stirred solution of 5'-dimethoxytrityl (DMT) 2'-deoxynucleoside [1 g, N^6-benzoyl (bz) dA, N^4-bz dC, N^2-bz dG, or dT] and $(iPr)_2NEt$ (2 mL) in CH_2Cl_2 (10 mL) at room temperature, and stirring was continued for 15 min. The resultant mixture was dissolved in CH_2Cl_2 (30 mL), extracted with ice-cold saturated aq $NaHCO_3$ (50 ml x 2), and then dried over anhyd $MgSO_4$. The concentrated filtrate was placed under a high-vacuum for 2 h to afford a "foamy" product which was dissolved in EtOAc (3 mL) and then precipitated by addition of the EtOAc solution to pentane at -70 °C. The filtered precipitate was dried over P_2O_5/KOH under a high-vacuum to give the 5'-DMT O-ethyl phosphoramidite derivatives of dA^{Nbz}, 750 mg, 59%; dC^{Nbz}, 650 mg, 51%; dG^{Nbz}, 955 mg, 76%; and dT, 740 mg, 56%. EI-MS did not show detectable parent ions, although expected fragment-ions were found: A-amidite, m/z, 303 (DMT), 240 (A^{Nbz} + 2H), 105 (bz); C-amidite, m/z 303 (DMT), 216 (C^{Nbz} + 2H), 105 (bz); G-amidite, m/z 303 (DMT), 105 (bz); T-amidite, m/z 527 [parent-ion minus $OP(OEt)N(iPr)_2$], 303 (DMT), 127 (Thy). ^{31}P NMR (δ_P, ppm, in CH_2Cl_2 with 10% v/v C_6D_6): A-amidite, 153.1, 152.8,

47:53; C-amidite, 153.3, 152.9, 49:51; G-amidite, 155.8, 155.5, 37:63; T-amidite, 153.1, 152.6, 56:44.

Synthesis of 3'-O-(2'-Deoxyadenosyl)-5'-O-thymidyl-O-ethyl-phosphate, d[A(Et)T], and Separation of Diastereomers

5'-HO-Thy bound to long-chain alkylamine CPG (Vega, 1 μmol) was manually treated[22] with a solution of 5'-DMT-dA^{Nbz}-3'-O-ethyl-N,N-diisopropyl-phosphoramidite (17 mg, 20 μmol) and 1H-tetrazole (4.2 mg, 60 μmol) for 3 min. Oxidation, detritylation, cleavage from support (conc. NH_4OH, 1 h, 25 °C), and then base-deprotection (conc. NH_4OH, 48 h, 25 °C) was followed by purification and diastereomer separation by HPLC: μBondapak, CH_3CN-H_2O = 15:85, 3.5 mL/min, fast-eluted d[A(Et)T], 18.5 min (7.5 OD_{260}-units,36% yield), slow-eluted d[A(Et)T], 20 min (5 OD_{260}-units, 24% yield).

Synthesis of 3'-O-(2'-Deoxyadenosyl)-5'-O-thymidyl-O-ethyl-phosphorothioate, d[A_S(Et)T], and Separation of Diastereomers

A 4-μmol scale synthesis was carried out as described above for d[A(Et)T] with the exception that the phosphite triester was reacted with a saturated solution of elemental sulfur in lutidine (1 mL, 16 h, 25 °C). HPLC: μBondapak, CH_3CN-H_2O = 20:80, 3.5 mL/min, fast-eluted d[A_S(Et)T] 31 min (14 OD_{260}-units, 17% yield), slow-eluted d[A_S(Et)T], 33.5 min (20 OD_{260}-units, 24% yield).

Conversion of d[A_S(Et)T] into 3'-O-(2'-Deoxyadenosyl)-5'-O-thymidyl-phosphorothioate, d(A_ST)

To separate solutions of each diastereomer of d[A_S(Et)T] (3 OD_{260}-units) in CH_3CN (100 μL) was added a mixture of PhSH-Et_3N-dioxane (1:2:2 v/v, 100 μL). In each case the reaction mixture was kept at 50 °C for 6 h before addition of 0.8 M aq HOAc (200 μL), extraction with CH_2Cl_2 (1 mL x 3), and then evaporation of the aqueous layer in a vacuum-centrifuge. The resultant residues were analyzed by HPLC using μBondapak and 0-30% CH_3CN in H_2O at 1%/min, 3.5 mL/min: fast-eluted d[A_S(Et)T] gave slow-eluted (S_p)-d(A_ST)[10] (11 min), and slow-eluted d[A_S(Et)T] gave fast-eluted (R_p)-d(A_ST)[10] (10.5 min), which were identified by co-injection with a synthetic sample of diastereomerically pure d(A_ST).[10]

Stereochemical Correlation of the Diastereomers of d[A_S(Et)T] and d[A(Et)T]

To separate solutions of each diastereomer of d[A_S(Et)T] (1 OD_{260}-unit) in CH_3CN-H_2O (1:1 v/v, 100 μL) was added 30% aq H_2O_2 (10 μL) at room temperature. In each case the reaction mixture was periodically analyzed using HPLC (μBondapak, CH_3CN-H_2O = 15:85, 3.5 mL/min), which established that after 48 h there was 50-60% conversion of fast- and slow-eluted d[A_S(Et)T] into slow- and fast-eluted d[A(Et)T], respectively. The identity of each

product was confirmed by co-injection with a synthetic sample of diastereomerically pure d[A(Et)T].

Stereochemistry of the Oxidation of Dinucleoside O-Isopropyl Phosphorothioates with Hydrogen Peroxide

To diastereomerically enriched solutions of the O-isopropyl (iPr) phosphorothioates (R_p)- and (S_p)-d[T_S(iPr)T][20] (R_p:S_p = 100:0, 85:15, and 0:100, 1 OD_{260}-unit) in CH_3CN-H_2O (1:1 v/v, 100 μL) was added 30% aq H_2O_2 (10 μL). In each case the reaction mixture was kept at room temperature for 48 h prior to HPLC analysis (μBondapak, CH_3CN-H_2O = 12:88, 3.5 mL/min), which showed <10% of the original amount of starting material and, within the error limits of peak integration (± 3%), completely stereoretentive formation of the isopropyl phosphotriesters, d[T(iPr)T]:[20] fast-eluted (R_p)-d[T_S(iPr)T] → slow-eluted (S_p)-d[T(iPr)T] (62 min); slow-eluted (S_p)-d[T_S(iPr)T] → fast-eluted (R_p)-d[T(iPr)T] (57 min). Each product was identified by co-injection with a synthetic sample of diastereomerically pure d[T(iPr)T].

Comparison of Procedures for Cleavage from Support, Deprotection, and Isolation of d[GGAA(Et)TTCC].

Trial with NH_4OH. An automated 1-μmol scale synthesis was ended so as to give the DMT derivative of the support-bound protected octanucleotide, which was cleaved from the support with conc. NH_4OH (1 h, 25 °C) and then deprotected by further ammoniolysis (60 °C, 10 h). HPLC analysis (gradient I) of the resultant crude product gave a 70:30 area-ratio for the octanucleotides, 5'-DMT-d(GGAATTCC) : 5'-DMT-d[GGAA(Et)TTCC], which were eluted as overlapped peaks at 17.2 and 17.8 min, respectively. The crude product was detritylated with aq HOAc (pH 2.7, 10 min, 25 °C), and HPLC analysis (gradient II) gave a 70:30 area-ratio for d(GGAATTCC) : d[GGAA(Et)TTCC]. The former compound was eluted at 8.0 min, and was identified by co-injection with an authentic sample.[10,29] The latter compounds, which were eluted at 11.5 and 12.7 min in an area-ratio of 55:45, respectively, were separately collected for identification by enzymatic digestion (vide infra).

Trial with Ethylenediamine-Ethanol (EDA-EtOH). An automated 10-μmol scale synthesis was ended so as to remove the DMT group from the support-bound protected octanucleotide. Five 0.5%-w/w portions of the N_2-dried support (1.5-1.7 mg) were each placed in a vial for treatment with freshly prepared EDA-EtOH[11c] (1:1 v/v, 500 μL) at room temperature for 1,2,3,4, and 10 h, after which H_2O (3 mL) was added and an aliquot (350 μL) of the resultant vortexed solution was immediately removed for HPLC analysis (gradient II). Partially deprotected species, which eluted after the diastereomers of d[GGAA(Et)TTCC],

were major peaks in the 1-h sample and minor peaks in the 2-h sample, while the 3, 4, and 10-h samples each showed approximately the same amount of fast- and slow-eluted d[GGAA(Et)TTCC], which had a 55:45 area-ratio.

Large-Scale Work-Up with EDA-EtOH and Product Isolation. The remaining support from the 10-μmol scale synthesis was treated with EDA-EtOH (2 mL) for 1.5 h at room temperature. Water (15 mL) was added, the mixture was vortexed, and the filtrate was then freeze-dried. Three equal portions of the oily residue were subjected to HPLC using a Zorbax ODS column and gradient II at 11.25 mL/min, which led to collection of the fast- (12.8 min) and slow-eluted (13.8 min) diastereomers of d[GGAA(Et)TTCC]. Pooled fractions were concentrated to dryness in a vacuum-centrifuge, and in each case the resultant residue was dissolved in 1 M NaCl (0.15 mL) and then precipitated by dropwise addition of EtOH (0.35 mL), which afforded 39 and 48 OD_{260}-units of the fast- and slow-eluted products, respectively, for NMR and UV studies (see accompanying paper) after lyophilization from D_2O (1 mL). The ethanolic supernatants were concentrated to dryness in a vacuum-centrifuge, and then desalted by elution with H_2O from a PD-10 column (Sephadex G-25 Medium, 1 mL fractions). Fractions 3-5 were pooled in each case to give an additional 51 and 15 OD_{260}-units of the fast- and slow-eluted products, which were used for hydrolysis and digestion experiments (vide infra). The combined, isolated yield of the products, after correction for the coupling efficiency (96%), was 24% assuming a molar extinction coefficient of 84,000.

Enzymatic Digestions of the Diastereomers of d[GGAA(Et)TTCC]

Tandem digestions with snake venom phosphodiesterase (SVPDE) followed by alkaline phosphatase were performed as described previously.[30] RP-HPLC analysis (gradient III) of the heat-denatured digestion mixture derived from the fast-eluted diastereomer gave 2 : 1 : 1 : 2 molar-ratios of dG (6.8 min) : dA (8.9 min) : dT (7.2 min) : dC (4.9 min), after correction of relative peak-areas using the digestion mixture derived from d(GGAATTCC) as a standard for calibration. In addition, 1 mol-equiv of (S_p)-d[A(Et)T] was eluted at 19.4 min. The same compositional data was obtained for the slow-eluted octamer; however, in this case, (R_p)-d[A(Et)T] was eluted at 19.7 min.

Co-Hydrolysis of d[GGAA(Et)TTCC] and d[GGAA(iPr)TTCC]

0.6 OD_{260}-units of fast- (12.9 min, gradient III) and 0.2 OD_{260}-units of slow-eluted (13.7 min) d[GGAA(Et)TTCC] were combined with 0.8 OD_{260}-units of a 1:1 mixture of fast- (13.7 min) and slow-eluted (14.4 min) d[GGAA(iPr)TTCC],[20] and each of the resultant mixtures was dissolved in 1 mL of sodium borate buffer (1 M, pH 7.4). Aliquots (50 μL) were removed after 0, 0.5, 1, 3, 17.5,

and 62.5 h of heating at 70 °C for analysis by HPLC (gradient III), which showed the gradual disappearance of the starting materials with concomitant formation of d(GGAATTCC) (11.3 min) as the only detectable product. A linear regression analysis of $\ln[(\text{peak-area})_t/(\text{peak-area})_o]$ vs. t for fast-eluted d[GGAA(Et)TTCC] gave $k = -7.8 \times 10^{-5}$ min^{-1} ($r = 0.957$), $\tau_{1/2} = 147$ h (29% total reaction); slow-eluted d[GGAA(iPr)TTCC] gave $k = -3.0 \times 10^{-4}$ min^{-1} ($r = 0.995$), $\tau_{1/2} = 51$ h (70% total reaction). The other two reactants co-eluted, and their rates of disappearance were not determined.

The procedure described above was repeated using 1 mL of 0.1 N NaOH and 0, 0.5, 1, 2.1, 3, and 4-h aliquots: fast-eluted d[GGAA(Et)TTCC] gave $k = -3.7 \times 10^{-3}$ min^{-1} ($r = 0.994$), $\tau_{1/2} = 4.5$ h (57% total reaction), and slow-eluted d[GGAA(iPr)TTCC] gave $k = -2.5 \times 10^{-3}$ min^{-1} ($r = 0.945$), $\tau_{1/2} = 4.6$ h (45% total reaction). Formation of the major product, d(GGAATTCC), which was eluted at 11.3 min and had a relative peak-area of 34%, was accompanied by co-formation of minor products, which were eluted at 10.0 min, 21%; 10.2 min, 16%; 11.1 min, 11%; and 11.5 min, 18%.

Synthesis, Isolation, and Characterization of d(GGAA$_S$TTCC)

An automated 0.2-μmol scale synthesis was performed using 20-sec coupling times for the O-β-cyanoethyl phosphoramidite reagents. During the cycle for addition of the first dA residue, capping was followed by program-interruption for manual column-washing with CH_3CN (3 mL), delivery of a saturated solution of S_8 in lutidine, column-heating (60 °C, 30 min), column-washing with CH_3CN (3 mL), and then continuation of the program; repetitive trityl yield = 73%. The NH_4OH-deprotected (60 °C, 10 h) 5'-HO product [4.5 OD_{260}-units, 49% (corrected)] was isolated by HPLC (gradient IV): d(GGAATTCC), 13.3 min, 25% (relative peak-area); d(GGAA$_S$TTCC), 16.0 min, 75%. Tandem digestion with SVPDE and alkaline phosphatase as described above for d[GGAA(Et)TTCC] was followed by HPLC analysis (gradient V, PRP-1 column): mol-ratios dC (6.5 min) : dG (9.8 min) : dT (10.7 min) : dA (12.3 min) : (S_p)-d(A$_S$T) (18.6 min) = 2 : 2: 1.5: 1.5: 0.5. Digestion with nuclease P1[30] followed by alkaline phosphatase gave the same results, with the exception that the undigested fragment was 0.5 equiv (R_p)-d(A$_S$T) (17.9 min); ratio $S_p/R_p \cong 1$.

Experiments with EcoRI Endonuclease

To an Eppendorf tube (1.5 mL) that contained 0.5-2.0 OD_{260}-units (3-12 nmol) of $\{d[GGAA(X)TTCC]\}_2$ (X = phosphodiester, ethyl phosphotriester, or phosphorothioate linkage) was added 20 μL of buffer, which contained 10 mM tris, 90 mM NaCl and 20 mM $MgCl_2$, and had been adjusted to pH 7.6 with 1 N HCl. A 20-μL aliquot of a stock solution of commercially available (Bethesda

Research Laboratories) EcoRI endonuclease [200 units (0.4 μg, 6 pmol dimer) in 50 mM tris-HCl (pH 7.2), 300 mM NaCl, 0.5 mM Na_2EDTA, 5 mM 2-mercaptoethanol, 5 mM Na_2EDTA, 0.2% v/v Triton X-100, 500 μg/mL BSA, 50% v/v glycerol] was combined with 160 μL of buffer in an Eppendorf tube (1.5 mL). After vortexing, both Eppendorf tubes were placed in a water bath at 16 °C, and the contents of the tubes were mixed after 4 h. Duplicate aliquots (40 μL) of the incubation mixture were removed periodically (5-75 h), added to 40 μL of 0.2 M EGTA (adjusted to pH 7.0 with 5 N NaOH), and stored at 4 °C for HPLC analysis using a PRP-1 column: for gradient VI, d(GG), 8.6 min; d(pAATTCC), 14.2 min; d(GGAATTCC), 16.2 min; d(AATTCC), 16.8 min; (S_p)- and (R_p)-d[GGAA(Et)TTCC], 19.7 and 21.0 min, respectively; for gradient VII, d(GG), 7.1 min; d(pAA_STTCC), 17.8 min; d(GGAA_STTCC), 20.5 min; d(AA_STTCC), 23.0 min. Peak-areas were used to calculate %-reactions. Experiments in the absence of BSA and at higher concentrations of enzyme (2,000 and 20,000 units) utilized a different commercial source of EcoRI (Boehringer Mannheim).

RESULTS AND DISCUSSION

Synthesis and Isolation of the Diastereomers of d[GGAA(Et)TTCC]

The phosphoramidite method[3,31] for DNA synthesis, which has been previously applied to the preparation of isopropyl phosphotriester modified oligonucleotides,[20,23] was extended to ethyl phosphotriester analogues of DNA. The required deoxynucleoside O-ethyl-N,N-diisopropylphosphoramidite reagents were obtained in non-optimized, isolated yields of 51-76% by conventional[28] reaction of chloro-ethoxy-diisopropylaminophosphine with 5'-DMT derivatives of dA^{Nbz}, dC^{Nbz}, dG^{NBz}, and dT. Protection of the N^2 position of dG with a benzoyl substituent rather than an isobutyryl[28] group led to improved solubility of the G-amidite reagent in CH_3CN at the 0.2-0.1 M concentrations (20 to 10-fold molar excess) which are generally employed.

A pilot synthesis of the modified octanucleotide, 5'-DMT-d[GGAA(Et)TTCC], was carried out on a 1-μmol scale with 10-fold molar excesses of O-β-cyanoethyl and O-ethyl phosphoramidite reagents. The selectivity of backbone-deprotection (decyanoethylation) with conc. NH_4OH at 60 °C for 10 h was evaluated by HPLC. 5'-DMT-d(GGAATTCC) was eluted at 17.2 min and overlapped with a minor peak at 17.8 min, which was assumed to be the ethylated product, based on its elution time relative to the isopropyl analogue, 5'-DMT-[GGAA(iPr)TTCC].[20] Detritylation of this mixture led to baseline-resolution of the presumptive fast- (11.5 min) and slow-eluted (12.7 min) ethylated products, which were present in near-equal amounts and were

well-separated from the less hydrophobic unmodified octamer (8 min). The presumptive ethylated products were collected and identified by tandem digestion with SVPDE and alkaline phosphatase, based on the molar ratios of the released deoxynucleosides and undigested d[A(Et)T]. In view of the reported[32] evidence for epimerization at phosphorus during 1H-tetrazole-catalyzed coupling of O-methyl phosphoramidites, no attempt was made to investigate the possibility of achieving stereospecific formation of the ethyl phosphotriester linkage using diastereomerically pure O-ethyl phosphoramidites.

A 10-μmol scale synthesis of d[GGAA(Et)TTCC] was then carried out using only a 5-fold molar excess of the phosphoramidite reagents, which gave an average coupling yield of 96%. To minimize the extent of de-ethylation, cleavage from the support and backbone/base-deprotection with EDA-EtOH at 25 °C was monitored by HPLC, which indicated that these reactions were complete after ca. 2 h. The fast- and slow-eluted diastereomers were collected by HPLC and further processed to give 90 and 63 OD_{260}-units of each product (36% combined yield), respectively, for the investigations described in the next sections, and for NMR and UV spectroscopic studies reported in the accompanying paper.

Assignment of R_p and S_p Configurations in d[GGAA(Et)TTCC]

The stereochemical correlation scheme for assigning the configurations of the ethyl phosphotriester linkages in the diastereomers of d[GGAA(Et)TTCC] is outlined in Fig. 1. Tandem SVPDE and alkaline phosphatase digestion of the fast- and slow-eluted diastereomers gave 2:1:1:2 molar ratios of dG:dA:dT:dC and 1 mol-equiv of undigested fast- and slow-eluted d[A(Et)T], respectively. The resistance of d[A(Et)T] to hydrolysis by SVPDE was expected based on results reported[18] for d[G(Et)A]. The fast- and slow-eluted diastereomers of d[A(Et)T] were also obtained by stereospecific oxidation of slow- and fast-eluted O-ethyl phosphorothioates, d[A_S(Et)T], respectively, using 3% H_2O_2 in CH_3CN-H_2O at 25 °C for 48 h. The diastereomers of d[A_S(Et)T] were, in addition, regio- and stereospecifically de-ethylated with PhSH-Et_3N in dioxane at 50 °C for 6 h to give fast- and slow-eluted (R_p)- and (S_p)-d(A_ST), respectively, whose absolute configurations at phosphorus were previously assigned[10] on the basis of their susceptibility or resistance to cleavage by SVPDE and nuclease P1: cleavage of dinucleoside phosphorothioates by SVPDE is R_p-selective,[33] whereas nuclease P1 is S_p-selective.[34] Demethylation of the phosphotriester moiety in 5'-DMT-d[G^{Nibu}(Me)A^{Nbz}]C(O)CH_2OMe with PhSH-Et_3N has been reported[13] to proceed with retention of configuration at phosphorus,

which is mechanistically reasonable for both this compound and d[A_S(Et)T]. It therefore follows that the slow- and fast-eluted diastereomers of d[A_S(Et)T] have the $\underline{R}_p$ and $\underline{S}_p$ configurations, respectively. To complete the stereochemical correlation scheme, it was necessary to determine whether the oxidation of d[A_S(Et)T] to d[A(Et)T] with H_2O_2 proceeded with retention or inversion of configuration at phosphorus. Previous investigations[20] of the stereochemistry of the oxidation of thiono phosphotriesters with m-chloroperbenzoic acid (mCPBA) to give phosphotriesters have found retention of configuration, viz., ($\underline{R}_p$)- and ($\underline{S}_p$)-d[T_S(iPr)T] gave ($\underline{S}_p$)- and ($\underline{R}_p$)-d[T(iPr)T], respectively. The compounds ($\underline{R}_p$)- and ($\underline{S}_p$)-d[T_S(iPr)T] were therefore reacted with H_2O_2 under the same conditions used for the oxidation of d[A_S(Et)T], and the products were found to be ($\underline{S}_p$)- and ($\underline{R}_p$)-d[T(iPr)T], respectively. This established that the H_2O_2-mediated oxidation of d[T_S(iPr)T] and, by extension, d[A_S(Et)T] occurred with retention of configuration at phosphorus.[35] The fast- and slow-eluted diastereomers of d[A(Et)T] and their corresponding progenitors, fast- and slow-eluted d[GGAA(Et)TTCC], therefore have the $\underline{S}_p$ and $\underline{R}_p$ configurations, respectively.

The stereochemical assignments for ($\underline{R}_p$)- and ($\underline{S}_p$)-d[GGAA(Et)TTCC] were independently established by measurement of proton-proton nuclear Overhauser effects in the B-form of the duplex, {d[GGAA(Et)TTCC]}$_2$ (see accompanying paper), which in turn firmly established the stereochemical assignments for the corresponding, enzymatically excised fragments, ($\underline{R}_p$)- and ($\underline{S}_p$)-d[A(Et)T].

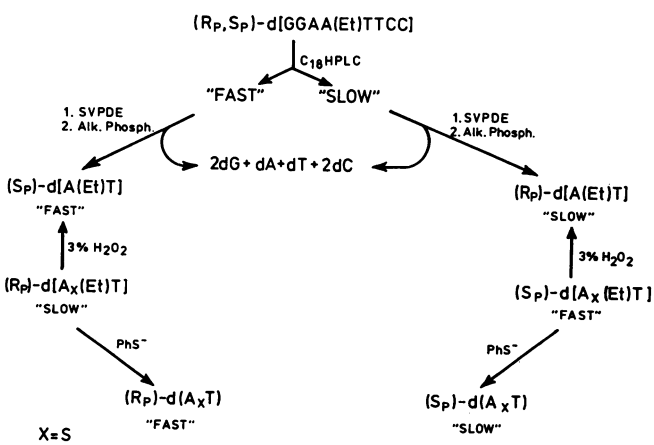

Fig. 1. Scheme for the configurational correlation between ethyl phosphotriester (Et) and phosphorothioate (X=S) modified oligonucleotides (see text for details); the use of phosphoroselenoates (X = Se) will be reported elsewhere (Stec, W.J., unpublished work).

This independent, NOE-derived configurational assignment for d[A(Et)T] together with the aforementioned evidence for retention of stereochemistry during both the oxidation of d[A_S(Et)T] with H_2O_2 (or mCPBA), and the de-ethylation of d[A_S(Et)T] with thiophenolate anion, provided an entirely new line of stereochemical evidence to support the $\underline{R}_p$- and $\underline{S}_p$-selectivity of SVPDE[33] and nuclease P1,[34] respectively, toward cleavage of dinucleoside phosphorothioate linkages.

The presently reported use of H_2O_2 rather than mCPBA for oxidation of O-ethyl phosphorothioate modified oligonucleotides has the advantage of substantially lessening troublesome secondary reactions between mCPBA and the desired, oxidized oligonucleotide products (data not reported). The major advantage of using PhSH-Et_3N rather than KSeCN[20] for de-ethylation of d[N(Et)N'] is that much lower temperatures (50 °C vs. 120 °C) are employed, which allows more reliable applications to higher oligomers, as was recently exemplified by de-ethylation of the individual diastereomers of d[G(^{17}O, Et)GAATTCC] with PhSH-Et_3N to give the isotopomers of d[G(^{17}O)GAATTCC].[22] It was found[22] that treatment of this ^{17}O-labeled, ethylated octanucleotide with PhSH-Et_3N in dioxane at 50 °C for 6 h led to 50% de-ethylation with only 5% side-reaction, although longer exposure-time led to increased proportions of decomposed oligonucleotides. We therefore investigated the possibility of using regioselective hydrolysis of a phosphotriester linkage as an alternative to de-alkylation by either PhSH-NEt_3 or KSeCN. Co-hydrolysis of the diastereomers of d[GGAA(R)TTCC], R = Et and iPr in borate buffer at pH 7.4, 70 °C was monitored by HPLC, and disappearance of starting materials was found to obey a pseudo-first-order rate law, with $\tau_{1/2}$ = 147 h for R = Et and $\tau_{1/2}$ = 51 h for R = iPr. The only product detected by RP-HPLC was the de-alkylated octanucleotide, d(GGAATTCC). By contrast, co-hydrolysis of these phosphotriesters in 0.1 N NaOH at 70 °C gave $\tau_{1/2}$ = 4.5 h for R = Et and $\tau_{1/2}$ = 4.6 h for R = iPr, with the appearance of de-alkylated octanucleotide accompanied by co-formation of 4 side-products, presumably due to other modes of phosphotriester cleavage.[16c,19] These results demonstrated that hydrolysis of ethyl and isopropyl phosphotriester modified oligonucleotides under neutral conditions was highly regioselective, leading to clean de-alkylation, and suggested that rate-limiting cleavage of the alkyl C-O bond occurred by an S_N1-type mechanism ($\underline{k}_{iPr}$ = 3 $\underline{k}_{Et}$).

Experiments with EcoRI Endonuclease

Incubations of EcoRI endonuclease with the duplexes {d[GGAA(X)TTCC]}$_2$, X = phosphodiester (PO), ethyl phosphotriester (POEt), and phosphorothioate

(PS), were carried out at 16 °C, which was *ca.* 10 °C below the T_m values for these duplexes in the 0.13 M tris-NaCl-$MgCl_2$ buffer-salt medium, although preliminary kinetic measurements at 16 °C and 25 °C indicated that the rates of cleavage for X = PO were roughly comparable, due in part to the fast ss/ds equilibrium at 16 °C relative to the slow ds hydrolysis (*vide infra*). Preliminary studies with X = PO also showed that a pronounced, initial lag-phase was largely abolished by pre-equilibration of the enzyme in the presence of Mg^{2+} for 4 h at 16 °C before adding the duplex. In the case with X = PO, at 60, 30, and 15 μM duplex with 30 nM enzyme-dimer (200 units), HPLC analysis of aliquots removed after 31 h of incubation revealed 22, 43, and 80% reaction, respectively, which indicated that the reaction was zero-order in substrate and had a maximal velocity of *ca.* 7 nM/min (turnover number = 0.004). After *ca.* 50% hydrolysis of the duplex, there was gradual disappearance of d(pAATTCC) with concomitant formation of d(AATTCC) due to a phosphatase activity. All of the aforementioned experiments employed enzyme in the presence of 0.7 μM BSA (preservative) and 10% v/v glycerol; however, the possibility of either inhibition[36] by BSA or "deactivation" by glycerol was ruled out by control studies using either added BSA (70 μM) or no BSA with less glycerol (1% v/v).

The doubly-modified duplex with X = PS (15 μM), as a mixture of $\underline{R}_p$-$\underline{R}_p$, $\underline{R}_p$-$\underline{S}_p$, and $\underline{S}_p$-$\underline{S}_p$ diastereomers, was digested in parallel with the unmodified duplex, X = PO. Analysis of duplicate aliquots removed after 6, 18, and 30 h indicated that the averaged, maximal velocities were 5.1 ± 0.5 and 7.8 ± 0.7 nM/min for X = PS and PO, respectively. Thus, the presence of a phosphorothioate linkage between the A and T residues caused a *ca.* 30% decrease in the overall rate of cleavage. A 10-fold scale-up of this cleavage reaction was taken to 50% conversion, and the recovered (HPLC) d(GGAA$_S$TTCC) and d(pAA$_S$TTCC) were each stereochemically analyzed by separate digestion with SVPDE and nuclease P1 followed by alkaline phosphatase. The ratios of deoxynucleosides to ($\underline{R}_p$)- or ($\underline{S}_p$)-d(A$_S$T) indicated that the cleavage by EcoRI was *ca.* 70% selective for the $\underline{S}_p$ configuration.

The individual $\underline{R}_p$-$\underline{R}_p$ and $\underline{S}_p$-$\underline{S}_p$ duplexes with X = POEt (30 μM) were found to be totally resistant to cleavage by EcoRI endonuclease (30 nM), even during prolonged incubation (96 h) in the presence of a 100-fold increase in the amount of enzyme-dimer (3 μM). Co-incubation of X = PO (30 μM) with either the $\underline{R}_p$-$\underline{R}_p$ or $\underline{S}_p$-$\underline{S}_p$ ethylated duplex (30 μM) demonstrated that the latter compounds did not measurably inhibit cleavage of the unmodified duplex by the enzyme (30 nM).

CONCLUSIONS

The present studies have shown that a combination of O-ethyl and O-β-cyanoethyl phosphoramidite reagents can be employed for the solid-phase synthesis of ethyl phosphotriester modified oligonucleotides. The lability of the ethyl phosphotriester linkage toward de-ethylation required relatively mild conditions for selective backbone- and complete base-deprotection: e.g., either conc. NH_4OH at 25 °C for 2 days, as found previously,[17b,22] or EDA-EtOH at 25 °C for several hours, as reported here. In using the latter conditions, which are essentially the same as those developed for methanephosphonate analogues of DNA,[11c] one should be aware of the possibility of side-reactions of dC^{NBz} residues with EDA.[37]

The configurational correlation scheme outlined in Fig. 1 was verified by independent NMR measurements (see accompanying paper), which therefore provided an entirely new line of evidence to support the correctness of assignments of the chirality at phosphorus in dinucleoside phosphorothioates previously based on enzymatic procedures.[10,33,34,38] This correlation scheme is generally applicable, and we believe that it is more reliable than those based on either NMR chemical shifts, HPLC elution times, or CD spectra.[14,15,16b] Various biologically-related [17d,18,19] stereochemical aspects of ethyl phosphotriester modified oligonucleotides can be studied using this method for assignment of chirality at phosphorus.

The maximal velocity for EcoRI endonuclease-catalyzed cleavage between the G and A residues in the phosphorothioate-modified duplex, $[d(GGAA_STTCC)]_2$, was decreased by ca. 30% relative to the unmodified duplex, and there was selectivity in favor of the S_p configuration. While this rate-depression was consistent with the earlier conclusion,[39] based on chemical ethylation interference experiments, that the A_pT phosphate is a contact point for the enzyme, the kinetic effect is relatively small and the magnitude of possible conformational perturbations, which must be considered,[24] are unknown at this time. The S_p selectivity, which is opposite to that found[10] for $[d(G_SGAATTCC)]_2$, suggests that the inferred[39] phosphate-protein interactions at the A_pT and G_pG positions have different stereochemical features which in each case leads to discrimination of the diastereotopic phosphate oxygens, however, here too the interpretations are complicated by possible conformational and electronic differences that result from substitution of $O{=}P{-}O^-$ by $O{=}P{-}S^-$.[9a]

The complete resistance of the $\underline{R}_p$-$\underline{R}_p$ and $\underline{S}_p$-$\underline{S}_p$ diastereomers of the doubly-modified ethylated duplex, {d[GGAA(Et)TTCC]}$_2$, toward cleavage by EcoRI endonuclease was consistent with the inferred[39] A_pT contact point. The chemical ethylation-interference experiments presumably[39] involved singly-modified DNA, which most likely had either the $\underline{R}_p$ or $\underline{S}_p$ configuration based on the known[19] stereochemical nonselectivity of 1-ethyl-1-nitrosourea. Detailed comparisons between synthetically and chemically ethylated DNA would therefore require mono-ethylated duplexes. In any event, it has been previously noted[24] that a major complication with ethylation interference is the superposition of charge-neutralization, steric, and conformational effects. The existence of such conformational differences in {d[GGAA(Et)TTCC]}$_2$ has been evidenced by 2D-NOE NMR measurements (see accompanying paper). We believe that the combined use of synthetic, stereochemically pure phosphorothioate- and ethyl phosphorotriester-modified oligonucleotides for detailed kinetic measurements and quantitative conformational analysis by NMR will provide important, new information concerning DNA recognition which is not available by chemical ethylation interference experiments alone.

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