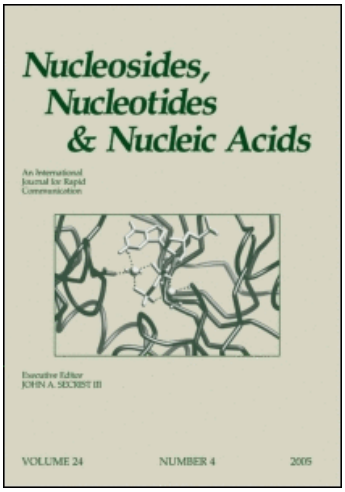


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Phosphate-Modified Oligonucleotides. The Synthesis, Stereochemistry and ECO Ri Endonuclease Substrate Ability of Decanucleotides d[GGGAATTCCC] Bearing Altered Internucleotide Phosphate Function Between A and A[1]

Maria Koziolkiewicz[a]; Bogdan Uznanski[a]; Wojciech J. Stec[a]
[a] Department of Bioorganic Chemistry, Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, Lodz, Boczna 5, Poland

To cite this Article Koziolkiewicz, Maria , Uznanski, Bogdan and Stec, Wojciech J.(1989) 'Phosphate-Modified Oligonucleotides. The Synthesis, Stereochemistry and ECO Ri Endonuclease Substrate Ability of Decanucleotides d[GGGAATTCCC] Bearing Altered Internucleotide Phosphate Function Between A and A[1]', Nucleosides, Nucleotides and Nucleic Acids, 8: 2, 185 — 200
To link to this Article: DOI: 10.1080/07328318908054167
URL: http://dx.doi.org/10.1080/07328318908054167

NUCLEOSIDES & NUCLEOTIDES, 8(2), 185-200 (1989)

PHOSPHATE-MODIFIED OLIGONUCLEOTIDES. THE SYNTHESIS, STEREOCHEMISTRY AND Eco RI ENDONUCLEASE SUBSTRATE ABILITY OF DECANUCLEOTIDES d[GGGAATTCCC] BEARING ALTERED INTERNUCLEOTIDE PHOSPHATE FUNCTION BETWEEN A AND A [1]

Maria Koziolkiewicz, Bogdan Uznanski, Wojciech J.Stec*

Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, Department of Bioorganic Chemistry, 90-362 Lodz, Boczna 5, Poland

Abstract: An interaction between Eco RI endonuclease and decadeoxyribonucleotide GGGAATTCCC is followed by means of oligonucleotide analogues bearing modified internucleotide phosphate functions bridging both adenosine residues. While an O-alkyl group at this phosphate, despite the "side" of DNA alkylation, completely prevents DNA from hydrolysis, a phosphorothioate function replacing phosphate at the position between A and A moieties controls the hydrolysis in terms of the absolute configuration at phosphorus. The fact, that the Rp-isomer of d[GGGA(S)ATTCCC] possessing sulphur atom directed "inward" DNA is hydrolyzed by Eco RI endonuclease may indicate, that the pro-S oxygen at this particular phosphate is involved in an interaction with magnesium ion, a necessary factor for executive action of this endonuclease.

It is well established that Eco RI endonuclease recognizes the canonical sequence 5'...GAATTC...3' of duplex DNA and in the presence of magnesium cations cleaves the internucleotide bond between G and A leaving the fragments ...G and pAATTC ...[2] . The importance of the recognition sequence GAATTC and the influence of alteration of the bases within this sequence (Eco RI* activity) and in flanking positions on the substrate selectivity of this enzyme have been also extensively studied in a number of research establishments[3,4]. The concept of "alkylation interference" introduced by Gilbert[5] and applied in studies on Eco RI endonuclease by Modrich[6], emphasized the participation of internucleotide phosphates in interactions with this protein. It has been established that phosphates in positions marked with triangles (Fig.1) are involved in interactions with protein.

FIG.1

The striking feature of these findings is the observation, that alkylation of the phosphate binding both adenosine moieties does not interfere with DNA-protein interaction, which indicates that this particular phosphate is not involved in the process of recognition or binding of the protein. Although fundamental studies by Rosenberg *et al.*[7] and Jen-Jacobson *et al.*[8] increased the understanding of the process of DNA-Eco RI interaction, these, like those of Modrich[6], have been performed in the absence of magnesium ions, an essential factor for the process of DNA cleavage by this enzyme.

Being involved in the studies on the synthesis and stereochemistry of phosphate-modified oligonucleotide analogues, we have obtained the decamer d[GGGAATTCCC] (1) and two pairs of its analogues. One has an ethylated phosphate function between adenosines d[GGGA(O)(OEt)ATTCCC] (2) and the other one contains a phosphorothioate function in the same position d[GGGA(S)ATTCCC] (3). Since the replacement of one of two non-bridging oxygens at this particular phosphate with the ethoxy group (2) or with sulphur (3) expresses the stereogenicity of the phosphorus atom, diastereomeric species have been synthesized and the absolute configuration has been elucidated for each individual diastereomer of 2 and 3. The assignment of absolute configuration at the P-chiral atom in modified oligonucleotides allows one to establish the orientation of the alkyl or sulphur substituent in the DNA molecule. The modified linkage in B-DNA can have either an "inward" oriented alkyl (sulphur) substituent (Rp configuration), which is directed toward the major groove, or an "outward" oriented substituent (Sp configuration) which is directed toward the solvent. The spatial orientation of the substituent modifying the internucleotide bond may be important for DNA-Eco RI interactions which have been reported[6,7] to occur in the major groove of DNA. Diastereomers 2 have to be considered as DNA analogues with a cancelled negative charge at the phosphate in question, while diastereomers of 3,

although retaining the charge, due to the known susceptibility of oxygen to magnesium, possess a preselected "side" of interaction with the metal ion. It was expected, that experiments on the substrate activity of each diastereomer 2 or 3 for Eco RI endonuclease may clarify the function of this particular phosphate in the process of cleavage of internucleotide bond between G and A ("scissile bond") catalyzed by this enzyme.

EXPERIMENTAL

Synthesis of base-protected 5'-DMT-2'-deoxyribonucleoside 3'-(O-2-cyanoethyl-N,N-diisopropyl)phosphoramidites

The synthesis of these compounds was performed as described previously[9]

Synthesis of 5'-DMT-N -benzoyl-2'-deoxyadenosine 3'-(O-ethyl--N,N-diisopropyl)phosphoramidite

This reagent was synthesized according to our procedure presented previously[10]. Its analysis was performed by means of EI-mass spectrometry and ^{31}P-NMR. EI-MS detected the following fragment-ions: m/z, 303 (DMT), 240(A^{NBZ} +2H), 105(Bz). ^{31}P-NMR chemical shifts obtained for this compound were as follows: (ppm, in CH_2Cl_2 with 10% v/v C_6D_6, 85% H_3PO_4 as an external standard) 153.1, 152.8, 47:53.

Synthesis of 3'-O-(2'-deoxyadenosyl)-5'-O-(2'-deoxyadenosyl)-O-ethyl phosphate d[A(O)(OEt)A] (4) and the separation of diastereomers

5'-HO-dA^{NBZ} bound to LCA CPG (Vega, 1 μmol) was treated[11] on a commercially available Applied Biosystems Inc. column with a 220 μl acetonitrile 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. After oxidation [0.1M iodine solution in 2,6-lutidine/water/THF (10:1:40 v/v/v) 1 ml, 1 min] and detritylation [$CHCl_2COOH/CH_2Cl_2$ (3:97 v/v) 2 ml, 1 min.] steps, the cleavage of the synthesized dinucleotide from the support was carried out by treatment with 25% aq. NH_4OH for 2h at 25°C. Deprotection of the bases was achieved by additional treatment with 25%aq. NH_4OH for 48h at 25°C. Purification and separation of d[A(O)(OEt)A] into its diastereomers was performed by means of HPLC on a

ODS-Hypersil column (30 cm x 4.6 mm) under isocratic conditions, CH_3CN-H_2O (12:88), flow-rate of 1.5 ml/min. Diastereomer "fast"-4 was eluted at 8.6 min. and its counterpart "slow"-4 was eluted at 9.6 min.

Synthesis of 3'-O-(2'-deoxyadenosyl)-5'-O-(2'-deoxyadenosyl)-O-ethyl phosphorothioate d[A(S)(OEt)A] (5) and the separation of diastereomers

The synthesis of this compound was performed as described above for 4 with the following modification: regular oxidation by means of 0.1M iodine solution in 2,6-lutidine/water/THF (10:1:40) was replaced by treatment of the intermediate phosphite triester with a saturated solution of elemental sulphur in lutidine (1 ml, 16h, 25°C). After washing of the column content with pyridine (5 ml) and CH_3CN (3 ml) 5'-DMT d[A(S)(OEt)A] was cleaved from the support by treatment of the column contents with 25% aq.NH_4OH for 2h at 25°C. Additional treatment of the liberated 5'-DMT 5 with 25% aq.NH_4OH (for 48h at 25°C) was used to deprotect the bases. Separation into diastereomers of 5'-DMT d[A(S)(OEt)A] was achieved on an ODS-Hypersil column under isocratic conditions, CH_3CN-H_2O (42:58), flow-rate of 1.5 ml/min. The "fast"-isomer of the 5'-DMT derivative of d[A(S)(OEt)A] was eluted at 7.0 min, while the "slow"-isomer was eluted at 9.3 min. The detritylation was achieved by treatment of each diastereomer of 5'-DMT d[A(S)(OEt)A] with a solution of $CH_3CN/H_2O/CH_3COOH$ (30:40:30) (1 ml, 1h, 25°C). After detritylation the solutions of each diastereomer were evaporated and the residues were purified on an ODS-Hypersil column with 20% aq.CH_3CN. Under these conditions both diastereomers of d[A(S)(OEt)A] (5) were eluted at 6.75 min giving "fast"-derived 5 and "slow" -derived 5.

Conversion of d[A(S)(OEt)A] (5) into 3'-O-(2'-deoxyadenosyl)--5'-O-(2'-deoxyadenosyl) phosphorothioate d[A(S)A] (6)

To a solution of each individual diastereomer of d[A(S)(OEt)A] (1 A_{260} unit of substrate dissolved in 100 µl CH_3CN) a mixture of PhSH/Et_3N/dioxane (1:2:2 v/v, 100 µl) was added and the reaction mixture was kept at 50°C for 6h. Then 200 µl of a 0.8 M aqueous solution of CH_3COOH was added and the mixture was extracted with CH_2Cl_2 (3x1 ml). The aqueous layer was evaporated, and the resulting residue was dissolved in a solution of CH_3CN-H_2O (1:1 v/v), and analyzed by means of HPLC on an

ODS-Hypersil column with the linear gradient 0-30% CH_3CN aq., 1%/min at a flow-rate of 1.5 ml/min. Under these conditions "fast"-derived 5 gave "slow"-eluted d[A(S)A] (6) (9.2 min), and "slow"-derived 5 was converted to "fast"-eluted d[A(S)A] (8.0 min). Products of dealkylation with PhS^- were indentified by co-injection with genuine samples of d[A(S)A][12].

Conversion of d[A(S)(OEt)A] (5) into d[A(O)(OEt)A] (4)

To a solution of each diastereomer of 5 [1 A_{260} unit of substrate dissolved in 100 µl of CH_3CN-H_2O (1:1)] 10 µl of 30% H_2O_2 was added. After 48h at room temperature the reaction mixture was analyzed by means of HPLC using the conditions described above for the separation of 4. HPLC analysis proved the conversion of "fast"-derived 5 and "slow"-derived 5 to "fast"-eluted and "slow"-eluted d[A(O)(OEt)A] (4), respectively. The products of oxidation of d[A(S)(OEt)A] with H_2O_2 were identified by co-injection with genuine sample of d[A(O)(OEt)A].

Synthesis of diastereomers of d[GGGA(O)(OEt)ATTCCC] (2)

The synthesis was carried out under conditions of the standard protocol[11] using protected 2'-deoxynucleoside 3'-(O-2-cyanoethyl-N,N-diisopropyl)phosphoramidites with one exception: in the cycle following the formation of internucleotide O-(2-cyanoethyl)phosphate between A and T, the regular 5'-DMT $dAde^{NBz}$ 3'-[$P(OCH_2CH_2CN)(N\textit{i}\text{-}Pr_2)$] reagent was replaced by 5'-DMT $dAde^{NBz}$ 3'-[$P(OC_2H_5)(N\textit{i}\text{-}Pr_2)$][9]. After a standard work-up, the 5'-DMT derivative of decanucleotide 2 was obtained, and its further purification was performed by means of HPLC on Waters µBondapak C_{18} column (30 cmx7.8 mm) with the gradient 5-30% CH_3CN-0.1M triethylammonium bicarbonate (TEAB), pH 7.4 (exp.0.25) for 20 min, followed by isocratic separation, at a flow-rate of 3.5 ml/min. Under these conditions, the separation of 5'-DMT d[GGGA(O)(OEt)ATTCCC] into its diastereomers was achieved: "fast"-DMT 2 was eluted at 18.5 min and "slow"-DMT 2 was eluted at 20.0 min. Separated diastereomers were detritylated [by treatment with 20% aqueous solution of CH_3COOH (20 min, 25°C)], and then repeatedly purified on µBondapak C_{18} column with the linear gradient 5-30% CH_3CN-0.1M TEAB, pH 7.4, 1.25%/min at a flow-rate of 3.5 ml/min. It appeared that "fast"-DMT 2 gave "slow"-2 (retention time 11.0 min) and from "slow"-DMT 2 "fast"-2 (retention time 10.5 min) was obtained. Both

"fast"-DMT 2 and "slow"-DMT 2 contained *ca.* 15% of decamer DMT-1* , which was inseparable during the chromatography of 5'-DMT derivatives. Compound 1 was separated from the diastereomers of 2 after the detritylation step by means of HPLC.

Synthesis and separation of diastereomers of the decamer d[GGGA(S)ATTCCC] (3)

A 0.5 μmol scale synthesis was performed analogously as was described for decamer d[GGGA(O)(OEt)ATTCCC]. However, during the cycle, for addition of the second dA residue with the use of 2'-deoxyadenosyl 3'-(O-ethyl-N,N-diisopropyl)phosphoramidite, the capping step was followed by sulphuration with a saturated solution of elemental sulphur in 2,6-lutidine (1 ml, 16h, 25°C). After washing of the column with pyridine (5 ml) and CH_3CN (3 ml) the regular synthesis was continued. Standard cleavage from the support, and base-deprotection, gave the decamer d[GGGA(S)(OEt)ATTCCC] which was analyzed by means of HPLC as the 5'-DMT derivative, as described above for 5'-DMT d[GGGA(O)(OEt)ATTCCC] (2). Partially separated diastereomers were detritylated (20% aq. CH_3COOH, 20 min) and once more applied to the μBondapak C_{18} column. Under the conditions described above for the HPLC analysis of 5'-HO 2 complete separation of d[GGGA(S)(OEt)ATTCCC] diastereomers (7) was achieved: "fast"-7 (from "slow"-DMT-7) was eluted at 12.4 min and "slow"-7 (from "fast"-DMT-7) was eluted at 12.8 min. Dealkylation of each diastereomer of 7 was carried out with concentrated ammonia (0.5 ml of 25% aq.NH_4OH, 48h, 55°C) and 95% removal of the ethyl group was achieved. Extention of the time of ammoniolysis to 60h gave complete dealkylation of the substrate. The HPLC analysis, performed under conditions described for preparation of the 5'-HO derivative of 7, allowed us to obtain "fast"-derived 3 and "slow"-derived 3 (retention time 8.8 min).

T_m measurements

About 0.5-0.7 A_{260} unit of 1 and of each diastereomer of 2 and 3 were separately dissolved in the buffer containing 10 mM Tris-Cl (pH 7.6),

* Formed during the alkaline deprotection of nucleobase amino groups by means of 25% aq. NH_4OH.

TABLE 1. T_m values for decanucleotides 1, 2 and 3

compound no.	T_m [°C]
1	47.0
"fast"-2	46.5
"slow"-2	39.5
"fast"-derived 3	47.0
"slow"-derived 3	46.5

80 mM NaCl and 20 mM $MgCl_2$ (1 ml). The melting temperature (T_m) was measured spectrofotometrically using a Specord M40 (Carl-Zeiss, Jena) at λ_{max}=258 nm. The T_m values for decamer 1 and its analogues 2 and 3 are presented in Table 1.

Enzymatic digestions of d[GGGA(O)(OEt)ATTCCC] (2)

Each diastereomer of 2 (0.25 A_{260} unit) was independently dissolved in 200 µl of the buffer containing 0.1M Tris-Cl (pH 8.5) and 15 mM $MgCl_2$ and incubated with snake venom phosphodiesterase (SVPDE) (10 µg) for 12h, and then with alkaline phosphatase (AP) (1 µg) for 1h at 37°C. After the heat-denaturation of the digestion mixture, HPLC analysis on ODS-Hypersil column was performed. Undigested dinucleoside O-ethyl phosphate d[A(O)(OEt)A] was isolated and compared by co-injection with a genuine sample of this compound of known absolute configuration at the P-atom. The digest of "fast"-2 (obtained from "slow"-DMT 2) contained the "slow"-eluted isomer of d[A(O)(OEt)A] (4) of Sp absolute configuration, while in the digest of the "slow"-2 (obtained from "fast"-DMT 2) the "fast"-eluted isomer of 4 (Rp configuration) was present.

Enzymatic digestion of the diastereomers of d[GGGA(S)ATTCCC] (3)

Each diastereomer of 3 (0.25 A_{260} unit) was separately dissolved in 200 µl of the appropriate buffer for SVPDE and the digestion was carried out as described above. Independently, the digestion of each diastereomer was carried out with nuclease P1. About 0.25 A_{260} unit of the oligomer dissolved in 200 µl of the buffer containing 0.1M Tris-Cl (pH 7.2) and 1mM $ZnCl_2$ was incubated with nuclease P1 (1 µg) for 12h at 37°C and

then with alkaline phosphatase for 1h at 37°C. The heat-denaturated digestion mixtures were analyzed on an ODS-Hypersil column with a linear gradient 0-30% CH_3CN-H_2O, 1.0%/min at a flow-rate of 1.5 ml/min. Undigested isomers of d[A(S)A] were compared by co-injection with a genuine sample of d[A(S)A] of known absolute configuration, and by this means the absolute configuration at the P-atom in these oligomers was assigned. "Fast"-derived 3 contained the "fast" isomer of d[A(S)A] of Rp configuration, while "slow"-derived 3 contained the "slow" isomer of d[A(S)A] of Sp absolute configuration.

Digestion of oligomers with Eco RI endonuclease

About 0.5 A_{260} unit (3 nmol) of 1, 2 or 3 was dissolved in 400 µl of the buffer containing 10 mM Tris-Cl (pH 7.6), 80 mM NaCl and 20 mM $MgCl_2$. To this solution 10 µl aliquot of Eco RI endonuclease solution (purchased from Bethesda Research Laboratories) was added (100 units, 0.2 µg of the protein, 3 pmol of the protein in its dimer form). The 50 µl aliquots of the digestion mixture were removed periodically, heat-denaturated and analyzed on a µBondapak C_{18} column with a linear gradient 5-20% CH_3CN - 0.1M TEAB (pH 7.4) 1%/min at a flow-rate of 3.5 ml/min under conditions allowing quantitation. In all analyzed incubation mixtures one of two possible products was identified, namely trinucleotide d[GGG]. Elution times for the undigested substrates and the products were as follows:

d[GGGAATTCCC] (1) ⟶ d[GGG] (8.1 min) + d[pAATTCCC] (9.7 min)
(10.0 min)

d[GGGA(O)(OEt)ATTCCC] (2) ("fast"), ("slow") ⟶ no products
(10.5 min) (11.0 min)

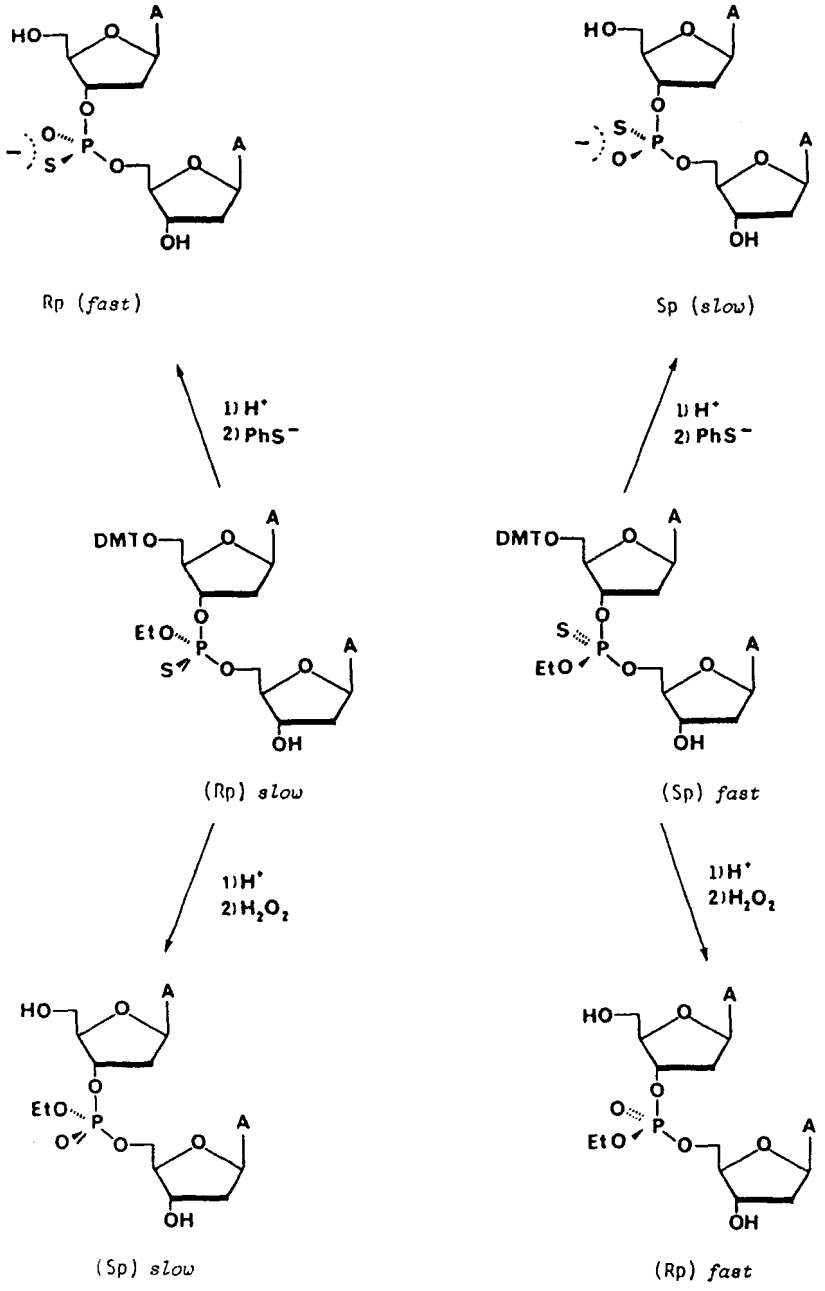
(Rp)-d[GGGA(S)ATTCCC] (3) ⟶ d[GGG] (8.1 min) + d[pA(S)ATTCCC] (10.3 min)
(10.3 min)

(Sp)-d[GGGA(S)ATTCCC] (3) ⟶ no products

RESULTS AND DISCUSSION

Synthesis of oligodeoxyribonucleotides bearing internucleotide O-ethyl phosphate or O-ethyl phosphorothioate function

Our approach to the synthesis of oligonucleotides containing an esterified internucleotide bond at a preselected position is based on the modification of the phosphoramidite method developed by Caruthers *et al.*[13]. For the synthesis of oligonucleotide bound *via* the 3'-oxygen of the "primer" nucleoside to a solid support we have used base-protected nucleoside 3'-(O-2-cyanoethyl-N,N-diisopropyl) phosphoramidites[9] and their 3'-(O-ethyl-N,N-diisopropyl)phosphoramidite analogues[10]. If the oxidation of the internucleotide O-ethyl phosphite function, usually performed by means of iodine in a lutidine/ water/THF (10:1:40) mixture, is replaced by sulphuration (0.4 M solution of elemental sulphur in 2,6-lutidine), then oligonucleotides bearing O-ethyl phosphorothioate functions at preselected positions are available. Cleavage from the solid support, removal of the 2-cyanoethyl phosphate protective groups, and base deprotection is achieved by treatment of the oligonucleotide bound to the support with 25% NH_4OH for 48h at 25°C. However, O-dealkylation (*ca.*15%) was also observed. This undesired dealkylation was avoided when the time of ammoniolysis was shortened to 24h. With the presented modifications (*vide supra*) we were able to get decamers 5'-DMT-d[GGGA(O)(OEt)ATTCCC] and 5'-DMT- d[GGGA(S)(OEt)ATTCCC]. Each product was isolated and separated into diastereomeric species by means of the RP-HPLC technique (see Experimental). The separation of the diastereomers of 5'-HO-d[GGGA(S)(OEt)ATTCCC] allowed us to convert each diastereomer of this compound into pure diastereomers of 5'-HO-d[GGGA(S)ATTCCC] by treatment with 25% NH_4OH at 55°C for 60h. Since the mixture of diastereomers of 3 appeared inseparable under RP-HPLC conditions[12], the approach presented in this paper offers a new way to prepare the diastereomers of the phosphorothioate analogues of oligonucleotides. It should be pointed out that the conversion d[GGGA(S)(OEt)ATTCCC] ⟶ d[GGGA(S)ATTCC] is also possible by means of PhSH/Et_3N/dioxane (50°C, 6h), but under these conditions dealkylation is not complete and there is the formation of some side products resulting from DNA chain cleavage. For the reasons presented below, we have also synthesized and separated into individual diastereomers the modified dinucleotides (4) and (5). Individual dia-



SCHEME 1.

stereomers of compound 5 were dealkylated by means of PhSH/Et_3N/dioxane, and, in this way, diastereomers of d[A(S)A] were obtained[12].

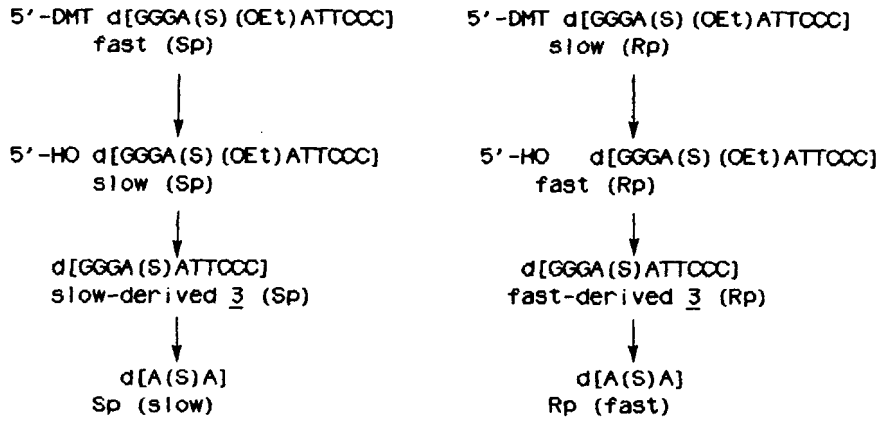
Assignment of absolute configuration at the stereogenic phosphorus atom in d[A(O)(OEt)A] (4) and d[A(S)(OEt)A] (5)

The stereochemical correlation for the assignment of the absolute configuration at the P-chiral centres in d[A(O)(OEt)A] and d[A(S)(OEt)A] is presented in Scheme 1. In this correlation, each diastereomer of d[A(S)(OEt)A] is independently converted into d[A(O)(OEt)A] and into d[A(S)A]. The conversion 5 ⟶ 4 was achieved by stereospecific oxidation using 3% H_2O_2 in CH_3CN-H_2O (1:1) at 25°C for 48h. Under these conditions 50-60% of the substrate 5 is oxidized to 4. Independently, each diastereomer of d[A(S)(OEt)A] was exposed to the action of thiophenol/Et_3N/dioxane solution (1:2:2) at 50°C for 6h[12]. This procedure allowed us to obtain the individual diastereomers of d[A(S)A], of known absolute configuration on the basis of their resistance and susceptibility to the action of SVPDE and nuclease P1. The (Rp)-isomer of d[A(S)A] (and other dinucleoside phosphorothioates) is resistant to the action of nuclease P1, while the (Sp)-isomer is resistant to the action of SVPDE[14,15]. Since both reactions, PS-PO conversion[10,16] and phosphotriester dealkylation by means of PhSH/Et_3N [17], are known to proceed with retention of configuration at the phosphorus atom, it was possible to correlate, by HPLC, the absolute configurations at phosphorus in 4 and 5 with those of each diastereomer of d[A(S)A].

Absolute configuration assignment in d[GGGA(O)(OEt)ATTCCC]

The assignment of the absolute configurations at the stereogenic P-atom centres in "fast"- and "slow"-eluted diastereomers of d[GGGA(O)(OEt)ATTCCC] (2) was performed after tandem SVPDE and alkaline phosphatase digestion of each diastereomer of 2. Under the action of SVPDE, only phosphodiester bonds were cleaved, and from the enzymic digest of each diastereomer of 2, the corresponding "fast"- or "slow"-eluted 4 was recovered by means of RP-HPLC. Their identification was ascertained by co-injection with a genuine sample of 4 prepared independently.

$$d[GGGA(O)(OEt)ATTCCC] \xrightarrow[2.AP]{1.SVPDE} d[A(O)(OEt)A] + 3G + 2T + 3C$$



SCHEME 2.

Thus, from the enzymic digest of the "fast"-2 diastereomer Sp ("slow")-4 was isolated, while Rp ("fast")-4 was obtained from the digest of "slow"-2. It is evident that the absolute configuration at phosphorus in "slow"-eluted 2 is Rp, while "fast"-eluted 2 has the Sp configuration.

Absolute configuration assignment at P-atom centre in d[GGGA(S)ATTCCC] (3)

The assignments of the absolute configurations at the stereogenic phosphorus atoms in the diastereomers of 3, were performed after dealkylation of 5'-HO-7 (see Experimental). Attempted degradation of each diastereomer of 3 by means of SVPDE and, independently, with nuclease P1, followed by alkaline phosphatase allowed us to assign the Rp configuration for "fast"-derived 3 and the Sp configuration for "slow"- derived 3, respectively (see Scheme 2).

Experiments with Eco RI endonuclease

Incubations of decamer d[GGGAATTCCC] (1) and its O-ethyl ester (2) and phosphorothioate (3) analogues with Eco RI endonuclease were performed at 18°C i.e. 20-30°C below the melting temperature for these duplexes in the incubation buffer. Therefore, it was expected, that under the experimental conditions employed, the modified oligomers 2 and 3 exist in duplex form[19]. Incubation of unmodified decamer 1 with the

enzyme for 24h resulted in the complete digestion of the substrate, and the formation of fragments d[GGG] and d[pAATTCCC]. The hydrolysis of the Rp isomer of 3 with Eco RI endonuclease, performed under identical conditions, resulted in 30% digestion of the substrate to d[GGG] and d[pA(S)ATTCCC], while the Sp isomer of d[GGGA(S)ATTCCC] was left intact (no traces of d[GGG] were detected). The lower rate of the enzyme-catalyzed hydrolysis of phosphorothioate analogues of DNA and oligonucleotides has been observed earlier[10,20]. The two-fold increase in Eco RI endonuclease concentration (from 200 units of the enzyme/1 A_{260} unit of the substrate to 400 units/1 A_{260} unit) did not cause any observable degradation of Sp-3. This result suggests that the presence of pro-S (in terms of absolute configuration) oxygen attached to the negatively charged internucleotide phosphate linking the two adenosine residues is the critical structural requirement for Eco RI endonuclease action. Incubations of both diastereomers of d[GGGA(O)(OEt)ATTCCC] with Eco RI restrictase, under conditions where the unmodified decamer was completely digested, allowed us to determine that neither of them was the substrate for this enzyme. Thus, the presence of the O-ethyl group at the internucleotide linkage between A and A caused complete resistance of the modified decamer to Eco RI endonuclease action.

In earlier studies it has been demonstrated that monophosphorothioate analogues of DNA bearing the canonical sequence ...GAATTC... are diastereoselectively digested by Eco RI endonuclease if phosphorothioate occupies preselected positions 2^{18}, 3^{21} or 5^{10}.

$$G^{1}\,G^{2}\,G^{3}\,A^{4}\,A^{5}\,T^{6}\,T^{7}\,C^{8}\,C^{9}\,C$$

It has been also reported, that oligonucleotides with phosphorothioates at positions 1,6,7 or 8[12] are substrates for Eco RI endonuclease, independently of the sense of chirality at the phosphorus of the phosphorothioate moiety. Remote control of the diastereoselectivity of Eco RI endonuclease towards DNA analogues bearing phosphorothioate at position 2 has been confronted with the conclusion[22] that the protection of DNA by phosphorothioate against restrictases is effective only if the phosphorothioate replaces the "scissile" phosphate bond[22]. Results presented in this report indicate that the remote control of the diastereoselec-

tivity of the enzymatic process operates also if the phosphorothioate replaces phosphate at the downstream position adjacent to the "scissile" bond.

Ethylation of phosphates at positions 1,2,3 or 5[1] and, as shown in this report, at position 4 (compound 2), effectively hampers the process of cleavage of the internucleotide phosphate at position 3. Ethylation at positions 7 and 8 causes dramatic decrease of the rate of release of the d[GGG] fragment without any diastereoselection, which has been observed if the phosphate at position 6 is esterified[1]. Most probably, such dramatic effect of ethylation at phosphate 7 and 8 on Eco RI digestion is caused by the influence of the esterified phosphates on the trimer d[GGA] placed directly across the dsDNA. The possibility of such interactions between DNA fragments which belong to different strands of the duplex and the enzyme was suggested by Jen-Jacobson[23]. Whether "ethyl-protection" results from the effect of steric hinderance and shielding of DNA from protein, or is due to conformational change of DNA, restricting the formation of a DNA-protein "tight interface", cannot be definitely concluded. However, it is evident that the ethylation of phosphate at position 4, independently on the "side" of ethylation, protects the DNA from cleavage of phosphate at position 3 by Eco RI restrictase. Whether this protection is due to negative charge cancellation, cannot be definitely said. The fact, that only the Rp diastereomer of 3 retains substrate activity towards Eco RI endonuclease, supports the hypothesis that the charge at position 4 is essential, and that this phosphate may be involved in the complexation of magnesium cation, necessary for the cleavage of phosphate at position 3. In agreement with Modrich's data[6], this particular phosphate may not be involved in direct interaction with protein. If the charge within the phosphorothioate moiety is not symmetrically distributed between the O and S atoms[24], only the (Rp)-diastereomer, bearing a charged oxygen directed outward of the DNA, is able to interact with magnesium ion, and the oligonucleotide can achieve a conformation appropriate for the executive action of the enzyme.

Results presented in this work may be considered as complementary to earlier studies on "alkylation interference"[6]. Our approach demonstrates the usefulness of synthetic DNA analogues bearing phosphorothioates or triester functions at preselected positions as tools for the

further elucidation of the DNA-protein interactions fulfilling the requirements of the presence of all components necessary for the executive enzyme action.

Acknowledgment: *Authors are indebted to Dr.L.Jen-Jacobson and Prof. F.Eckstein for providing their results prior to publication.*
This project was financially assisted by National Program CPBR-3.13.

REFERENCES

1. Preliminary results on Eco RI endonuclease activity toward DNA-triesters were presented at the VIIth International Round Table: Nucleosides, Nucleotides and Their Biological Applications, and published without experimental part in Nucleosides & Nucleotides, 6, 111 (1987)
2. J.Hedgpeth, H.M.Goodman, H.W.Boyer, Proc.Natl.Acad. Sci. USA, 69, 3448 (1972); P.Modrich, CRC Crit.Rev.Biochem., 13, 287 (1982).
3. J.M.Rosenberg, P.Greene, DNA, 1, 117 (1982).
4. S.E.Halford, N.P.Johnson, J.Grinsted, Biochem.J., 191, 581 (1980); K.L.Berkner, W.R.Folk, Anal.Biochem., 129, 446 (1983); J.Alves, A.Pingoud, W.Haupt, J.Langowski, F.Peters, G.Maass, C.Wolf, Eur.J.Biochem., 140, 83 (1984).
5. U.Siebenlist, W.Gilbert, Proc.Natl.Acad.Sci.USA, 77, 122 (1980)
6. A.-L.Lu, W.E.Jack, P.Modrich, J.Biol.Chem., 256, 13200 (1981)
7. Ch.A.Frederick, J.Grable, M.Melia, C.Samudzi, L.Jen-Jacobson, B.Ch.Wang, P.Greene, H.W.Boyer, J.M.Rosenberg, Nature, 309, 327 (1984); J.A.McClarin, Ch.A.Frederick, B.-Ch.Wang, P.Greene, H.W.Boyer, J.Grable, J.M.Rosenberg, Science, 234, 1526 (1986).
8. L.Jen-Jacobson, D.Lesser, M.Kurpiewski, Cell, 45, 619 (1986).
9. N.D.Sinha, J.Biernat, J.McManus, H,Koster, Nucl.Acids Res., 12, 4539 (1984).
10. K.A.Gallo, K.Shao, L.R.Phillips, J.B.Regan, M.KoziolKiewicz, B.Uznanski, W.J.Stec, G.Zon, Nucl.Acids Res., 14, 7405 (1986).
11. B.Uznanski, M.KoziolKiewicz, W.J.Stec, G.Zon, K.Shinozuka, L.G.Marzilli, Chem.Scr., 26, 221 (1986).
12. M.KoziolKiewicz, B.Uznanski, W.J.Stec, G.Zon, Chem.Scr., 26, 251 (1986).
13. M.H.Caruthers, Science, 230, 281 (1985).

14. R.F.Bryant, S.J.Benkovic, Biochemistry, 18, 2825 (1979); P.M.J.Burgers, F.Eckstein, D.H.Hunneman, J.Biol.Chem., 254, 7476 (1979); P.M.J.Burgers, B.K.Sathyanarayana, W.Saenger, F.Eckstein, Eur.J.Biochem., 100, 585 (1979).
15. B.V.L.Potter, B.A.Connolly, F.Eckstein, Biochemistry, 22, 1369 (1983).
16. W.J.Stec, A.Okruszek, J.Michalski, J.Org.Chem., 41, 233 (1976).
17. B.V.L.Potter, F.Eckstein, B.Uznanski, Nucl.Acids Res., 11, 7087 (1983).
18. M.Koziolkiewicz, W.Niewiarowski, B.Uznanski, W.J.Stec, Phosphorus and Sulfur, 27, 81 (1986).
19. C.A.Brennan, R.I.Gumport, Nucl.Acids.Res., 13, 8665 (1985)
20. F.Eckstein, Ann.Rev.Biochem., 54, 367 (1985)
21. B.A.Connolly, B.V.L.Potter, F.Eckstein, A.Pingoud, L.Grotjahn, Biochemistry, 23, 3443 (1984).
22. H.P.Vosberg, F.Eckstein, J.Biol.Chem., 257, 6595 (1982); F.Eckstein, Biochem.Soc.Trans., 614th Meeting, Oxford, p.204 (1986).
23. L.Jen-Jacobson, personal communication.
24. W.Hinrichs, M.Steifa, W.Saenger, F.Eckstein-in press

Received November 6, 1987.