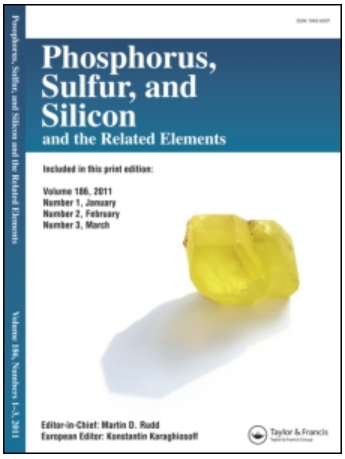

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

REMOTE CONTROL OF DIASTEREOSELECTIVITY OF DN-ASE II AND ENDONUCLEASE ECO RI TOWARDS PHOSPHOROTHIOATE ANALOGUES OF OLIGONUCLEOTIDES

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To cite this Article Koziołkiewicz, Maria , Niewiarowski, Wojciech , Uznanski, Bogdan and Stec, Wojciech J.(1986) 'REMOTE CONTROL OF DIASTEREOSELECTIVITY OF DN-ASE II AND ENDONUCLEASE ECO RI TOWARDS PHOSPHOROTHIOATE ANALOGUES OF OLIGONUCLEOTIDES', Phosphorus, Sulfur, and Silicon and the Related Elements, 27: 1, 81 — 92
To link to this Article: DOI: 10.1080/03086648608072761
URL: http://dx.doi.org/10.1080/03086648608072761

Phosphorus and Sulfur, 1986, Vol. 27, pp. 81–92
0308-664X/86/2702–0081/$20.00/0

REMOTE CONTROL OF DIASTEREOSELECTIVITY OF DN-ase II AND ENDONUCLEASE Eco RI TOWARDS PHOSPHOROTHIOATE ANALOGUES OF OLIGONUCLEOTIDES

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(Rp)-Thymidyl 3′-(4-nitrophenyl phosphorothioate) ($T_{P(S)}NP$) is converted in the presence of DN-ase II into (Sp,Rp)-thymidyl (3′-5′)-thymidyl phosphorothioate 3′-(4-nitrophenyl phosphorothioate) ($T_{P(S)}T_{P(S)}NP$), while (Sp)-$T_{P(S)}NP$ under analogous conditions gives (Rp,Sp)-$T_{P(S)}T_{P(S)}NP$, but as a minor product only. The preponderant product was recognized as (Sp,Rp)-thymidyl (3′-5′) thymidyl phosphorothioate 5′-(4-nitrophenyl phosphorothioate) ($NP_{P(S)}T_{P(S)}T$). All phosphorothioyl transfer reactions occur with retention of configuration at phosphorus atoms, which speaks for a double-displacement process and involvement of phosphorothioylated enzymes as reactive intermediates. Nucleolytic activity of DN-ase II, characteristic for this enzyme with respect to natural oligonucleotides, is not observed if $T_{P(S)}NP$ are used as the substrates. However, the transferase activity is even extended for the (4-nitrophenyl phosphorothioate)-shift from 3′ to 5′-position of dinucleotide, but for diastereoisomer (Rp,Sp)-$T_{P(S)}T_{P(S)}NP$ only. Such "remote" control of diastereoselectivity of DN-ase II is not unique, because Endonuclease Eco RI exerts similar selectivity towards diastereoisomers of octanucleotide $G_{P(S)}GAATTCC$. (Rp)-isomer, but not (Sp)-counterpart is the substrate for the enzyme causing its degradation to (Rp)-$G_{P(S)}G$ and $_{P}AATTCC$.

The influence of absolute configuration at phosphorothioate in 5′-"adjacent" position to the "scissile" internucleotide phosphate bond is discussed in terms of conformational changes of oligonucleotide or diastereofacial character of DNA-protein interaction and importance of protein-adjacent phosphate charge-charge interactions.

INTRODUCTION

The formation and cleavage of phosphodiester bonds, being essential for the structure and function of nucleic acids, occur with participation of corresponding enzymes which facilitate the process of nucleophilic substitution at phosphorus. An intriguing question about the role of enzymes in these processes is usually considered in terms of single (eq. 1)—or double (eq. 2) displacement process.[1]

$$E + A—P \rightleftharpoons E \cdot A—P \overset{+B}{\rightleftharpoons} E \cdot A—P \cdot B \rightleftharpoons E \cdot A \cdot P—B \rightleftharpoons E + A + P—B \quad (1)$$

$$E + A—P \rightleftharpoons E \cdot A—P \overset{-A}{\rightleftharpoons} E—P \overset{B}{\rightleftharpoons} E—P \cdot B \rightleftharpoons E \cdot P—B \overset{-E}{\rightleftharpoons} P—B \quad (2)$$

Single displacement process involves the formation of binary complex E · A—P between an enzyme E and phosphodonor A—P. The forces stabilizing E · A—P are

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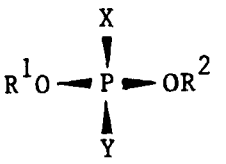
hydrogen bond-type interactions and coulombic charge-charge interactions. Binary complex E · A—P in the presence of acceptor molecule B (water or another nucleophile) is converted into ternary complex E · A—P · B, within which the rearrangement of covalent bonds occurs; covalent bond between A and phosphorus is broken and new covalent bond between phosphorus and B is formulated, resulting in formation of new ternary complex E · A · P—B which collapses reconstituting an enzyme E and producing the new phosphorylated molecule P—B.

Alternative double-displacement reaction also involves the primary formation of binary complex E · A—P, which dissociate to phosphorylated enzyme E—P and dephosphorylated donor A. Intermediary complex E—P is attacked by acceptor molecule B with formation of another intermediate E—P · B, which via phosphoryl transfer rearranges to E · P—B; its collapse regenerates enzyme E and releases phosphorylated acceptor B—P.

Apparently, these two modes of phosphoryl transfer are described by two different kinetic formalisms, but the other factor distinguishing between these two modes of enzyme–substrate interactions is the number of nucleophilic substitutions at phosphorus.

Since from the numerous studies on the process of nucleophilic substitution at phosphorus the general conclusion can be drawn that, with the few exceptions,[2] each act of nucleophilic substitution at phosphorus is accompanied with inversion of configuration at this stereogenic element, stereochemistry could be used as the complementary tool for elucidation of the mode of action of enzymes, because single-displacement process gives the product A—P with inverted configuration at phosphorus, while double-displacement process must be accompanied with retention of configuration on transferable phosphorus atom. This stereochemical approach to mechanistic studies on the enzyme-catalyzed phosphoryl transfer became feasible due to development of methodologies which allow the stereospecific synthesis of P-chiral phosphates (X = ^{17}O,^{18}O; Y = ^{16}O) or their phosphorothioate analogues (X = S, Y = O) and the quantitative assignment of the sense of chirality within the substrate AP* and product BP* molecules.[3,4]

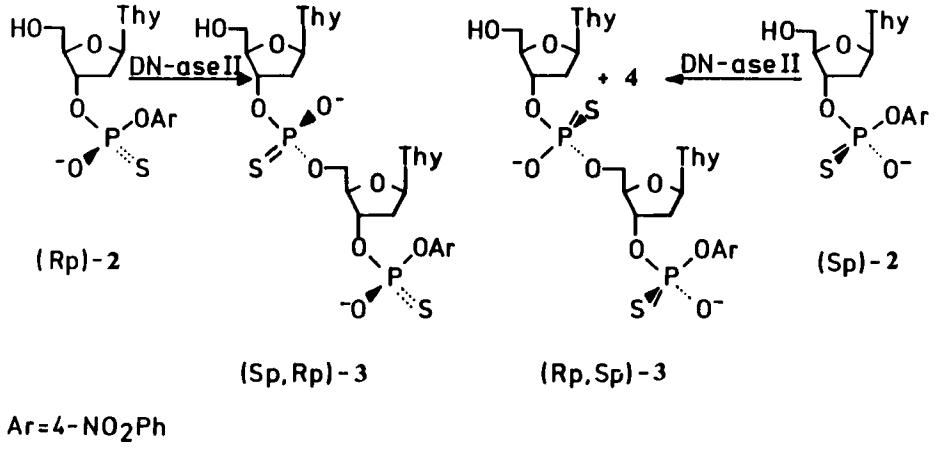


Historically, the first works on the stereochemistry of phosphoryl-transfer enzyme-catalyzed processes were performed with the use of phosphorothioates and were initiated by Eckstein and Usher in their classical work on pancreatic ribonuclease mode of action.[5] Further works from laboratories of Eckstein,[6] Benkovic,[7] and Frey[8] formulated the milestones for stereochemistry of enzyme-catalyzed phosphoryl-transfer processes, and kindled the creative thinking about even more close to natural products models for stereochemical studies, such as isotopomeric P-chiral alkyl |^{16}O,^{17}O,^{18}O| phosphates. Due to classical works of Knowles[9] and Lowe[10] the stereochemical approach to mechanistic studies on the molecular description of enzyme-catalyzed phosphoryl-transfer processes became

feasible, and practically unlimited. Examples of its creative applications, besides works mentioned above, are numerous and summarized in a number of excellent reviews[11,12] and monographs.[1,3,4] The most spectacular recent examples illustrating the potential of stereochemistry in evaluating the fate of biophosphates are the works of Tsai and associates on the metabolism of phospholipids[13] and Gerlt[14] and Eckstein[15] on the mechanism of adenylate cyclase catalyzed ATP → cAMP transformation.

ATTEMPTED DN-ase II-CATALYZED HYDROLYSIS OF DIASTEREOISOMERIC THYMIDYL 3′-(4-NITROPHENYL PHOSPHOROTHIOATE)

Achievements of this Laboratory in the synthesis of diastereoisomeric phosphoramidates, their stereochemical analysis and transformations into P-chiral phosphates and phosphorothioates, especially nucleoside cyclic 3′,5′-phosphorothioates, and their impact for the synthesis of isotopodiastereoisomeric counterparts, were summarized in earlier reviews.[4,16] In this paper we would like to report on the interaction of thymidyl 3′-(4-nitrophenyl phosphorothioates) (**2**)[17] with DN-ase II (E.C. 3.1.22.1). This enzyme was broadly used for degradation of DNA into deoxyribonucleoside 3′-phosphates.[18] It was of interest whether this enzyme interacts with DNA via single- or double-displacement process. Diastereoisomers of thymidyl 3′-(4-nitrophenyl phosphorothioate) ($T_{P(S)}NP$, **2**) were prepared via phosphorylation of 5′-monomethoxytritylthymidine with 0-(4-nitrophenyl)-*N*-phenyl-phosphoramidochloridate, chromatographic separation of diastereoisomers of 5′-monomethoxytritylthymidyl 3′-(4-nitrophenyl-*N*-phenylphosphoranilidate) (**1**) and their stereospecific conversion by means NaH/CS_2 into desired compounds **2**.[18] Absolute configuration at phosphorus was assigned by means of cyclisation of individual

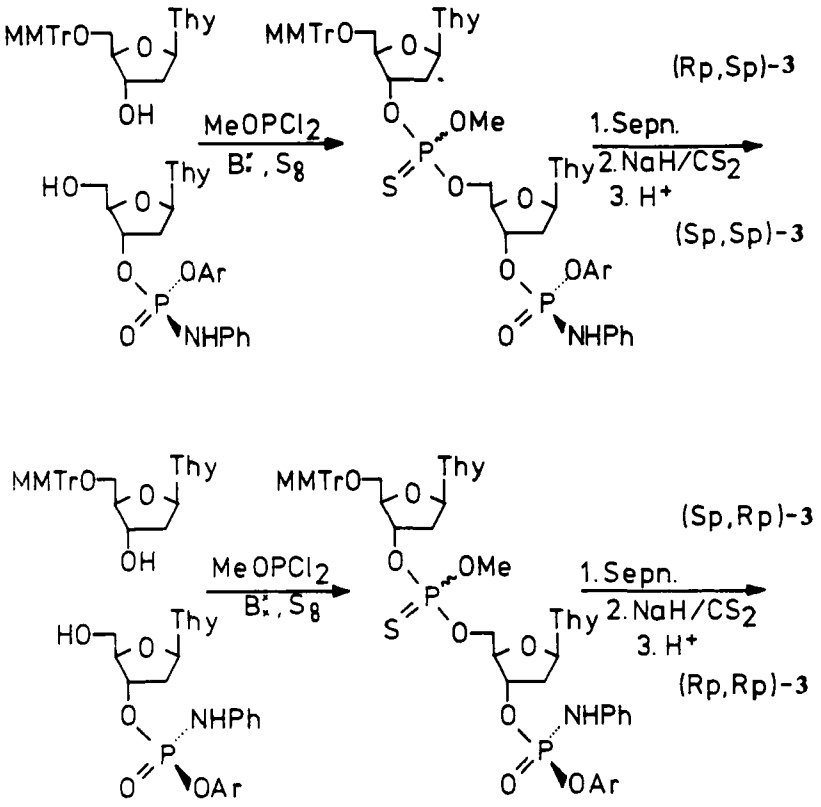


SCHEME 1

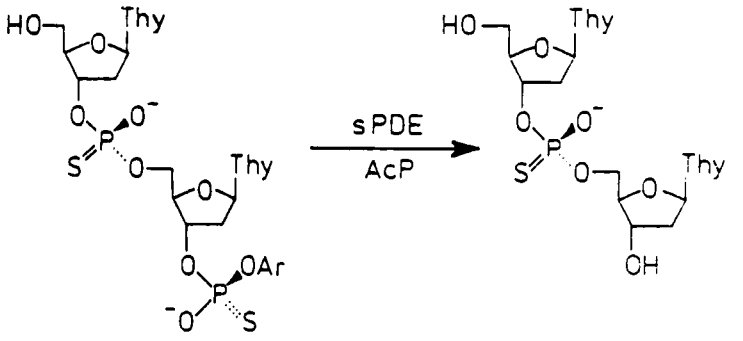
diastereoisomers of (**1**), after deprotection of 5′-hydroxy-function, into thymidyne cyclic (3′,5′)-phosphoranilidates.[19]

Attempted hydrolysis of (Rp)-**2** [0.012 mmol as its triethylammonium salt was dissolved in 0.05 sodium acetate buffer, pH 5.0 (2 ml) and DN-ase II (400 μg) was added; reaction mixture was incubated for 40 h at 37°C] in the presence of DN-ase II resulted, contrary to our expectations, in the formation of (Sp,Rp)-thymidyl (3′-5′)-thymidyl phosphorothioate 3′-(4-nitrophenyl phosphorothioate) ($T_{P(S)}T_{P(S)}NP$, **3**) while (Sp)-**2** gave (Rp,Sp)-**3**, but as a minor product only. The major product of attempted hydrolysis of (Sp)-**2** in the presence of DN-ase II was not identical with any of four diastereoisomers of **3** which were prepared in independent way, as indicated in Scheme 2. Absolute configurations in all four diastereoisomers of **3** at internucleotide phosphorus atom were assigned by means of enzymatic degradation of **3**[20] into thymidyl (3′-5′)thymidine phosphorothioate [$T_{P(S)}T$] and comparison of products with genuine samples of (Rp)- and (Sp)-$T_{P(S)}T$ prepared according to our original method.[21]

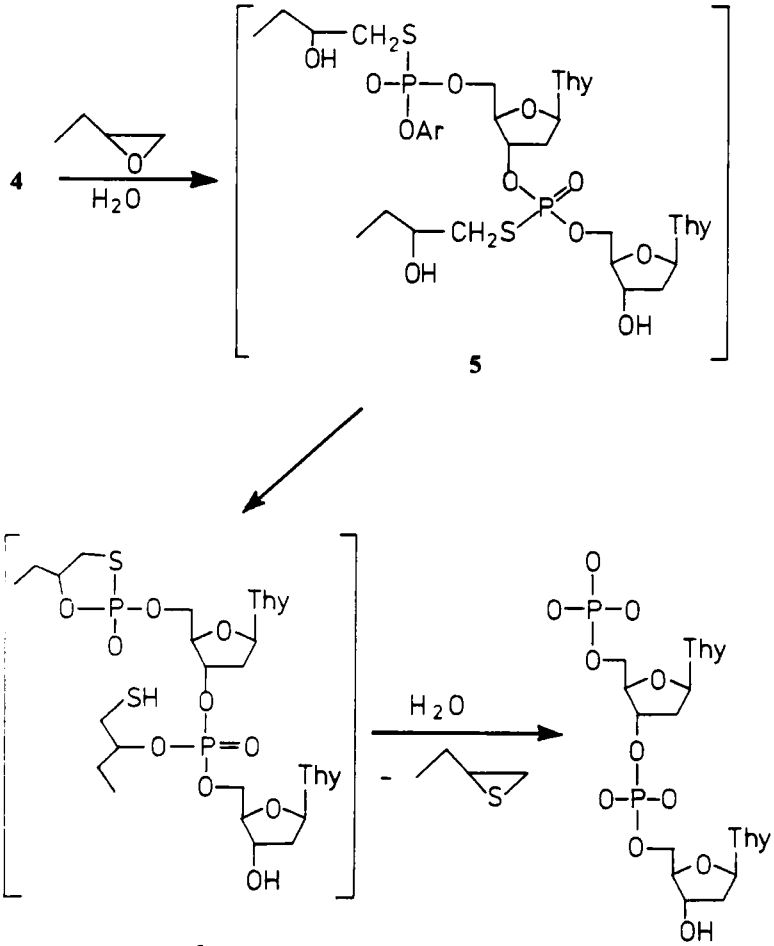
Because the compound **4**, an unknown second product of hydrolysis of (Sp)-**2** in the presence of DN-ase II, had identical ^{31}P- and ^{1}H-NMR spectra with (Rp,Sp)-**3**, an assumption was formulated, that it may be isomeric product of **3**, namely thymidyl (3′-5′)thymidyl phosphorothioate 5′-(4-nitrophenyl phosphorothioate) ($NP_{P(S)}T_{P(S)}T$, **4**).



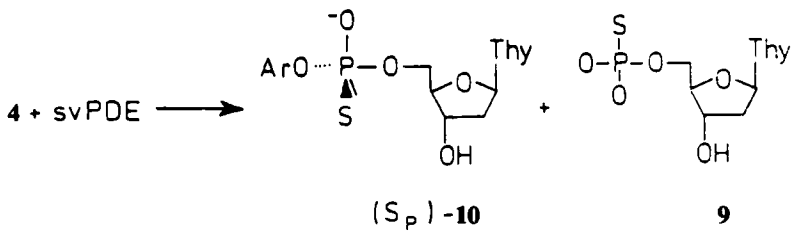
SCHEME 2



SCHEME 3



SCHEME 4



SCHEME 5

This hypothesis was confirmed by two independent routes. First one involved an oxidative hydrolysis of **4** with butane-1,2-oxide, the process originally observed in this Laboratory[22] which occurs with participation of intermediary products **5** and **6**.

The isolated compound **4**, when exposed on the action of butane-1,2-oxide/water gave thymidyl (3′-5′)thymidyl phosphate 5′-phosphate ($_pT_pT$, **7**), which by means alkaline phosphatase was further converted into thymidyl (3′-5′)-thymidine phosphate (T_pT, **8**). Another route involved the degradation of compound **4** with snake-venom phosphodiesterase, resulting in the formation of thymidyl 5′-phosphorothioate (**9**) and (Sp)-thymidyl 5′-(4-nitrophenyl phosphorothioate) [(Sp)-**10**].[19]

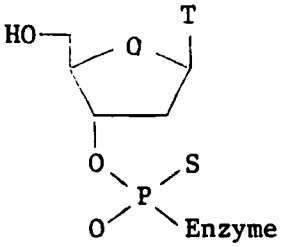
Since snake-venom phosphodiesterase is known to be stereoselective towards (Rp)-dinucleotidyl phosphorothioates, the analysis of the product resulting from both routes allowed us to confirm our hypothesis and establish the structure of **4** as (Sp,Rp)-**4**.

This unusual course of events forced us to perform another set of experiments: an isolated (Rp,Sp)-**3** was separately exposed for action of DN-ase II and the slow rearrangement of compound (Rp,Sp)-**3** → (Sp,Rp)-**4** was observed. Independently, each diastereoisomer of formerly synthesized **3** was exposed for an action of DN-ase II. While three remaining diastereoisomers of **3** were intact, only (Rp,Sp)-**3** was converted to (Sp,Rp)-**4**.[23]

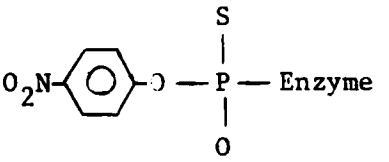
All observed facts can be rationalized as follows:

1. DN-ase II demonstrates phosphotransferase activity towards diastereoisomers of **2**.

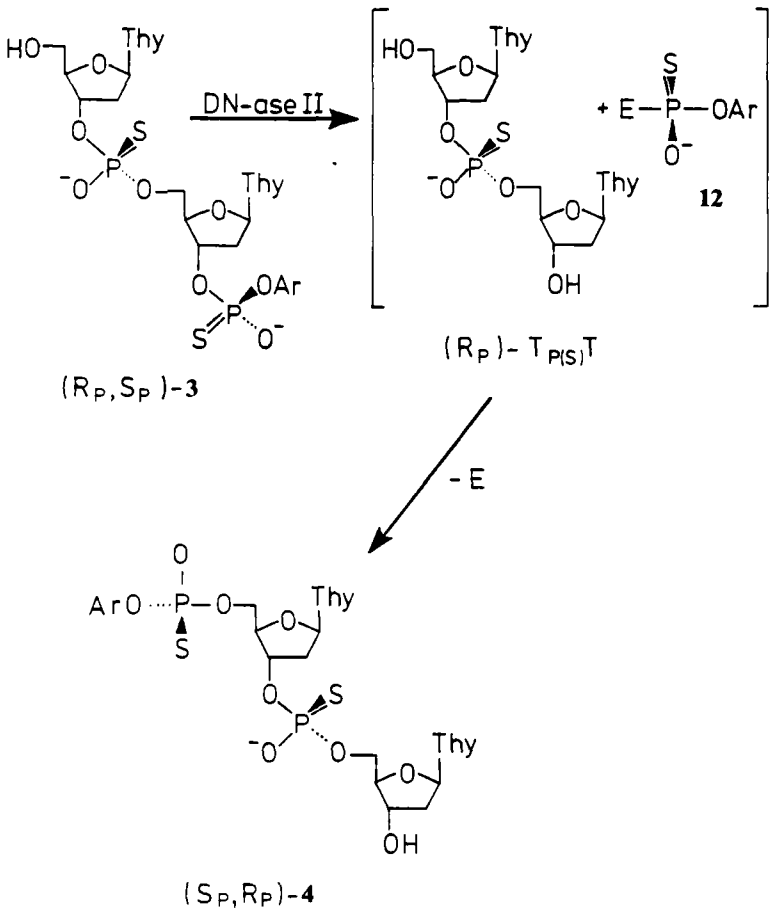
2. With respect to (Rp)-**2** reaction occurs with retention of configuration at phosphorus and formation of (Sp,Rp)-**3**. This result speaks for the transient formation of thymidyl 3′-phosphorothioylated enzyme **11** (*first inversion*) which undergoes nucleophilic attack by 5′-hydroxy group of the second substrate molecule (*second inversion*) and the formation of **3**.



11



12



SCHEME 6

3. (Sp)-**2** reacts in the same way as (Rp)-**2** but resulting compound (Rp,Sp)-**3** undergoes further attack by the enzyme at the terminal phosphorus atom with release of (Rp)-thymidyl (3′-5′)thymidine phosphorothioate, which then attacks the intermediate (**12**) by its 5′-hydroxyl group, and a final product (Sp,Rp)-**4** is formed. The 3′ → 5′ transfer of 4-nitrophenyl phosphorothioate moiety also occurs with retention of configuration (*double inversion*).

The most essential question may be raised, whether 3′ → 5′ phosphoryl transfer is catalyzed by the same enzyme, DN-ase II, or by the trace of another protein contaminating commercially available DN-ase II? At this moment we are not able to answer this question definitely, but it should be emphasized that an enzyme obtained from the two independent sources (Sigma and Miles Laboratories) have shown identical activities.[23]

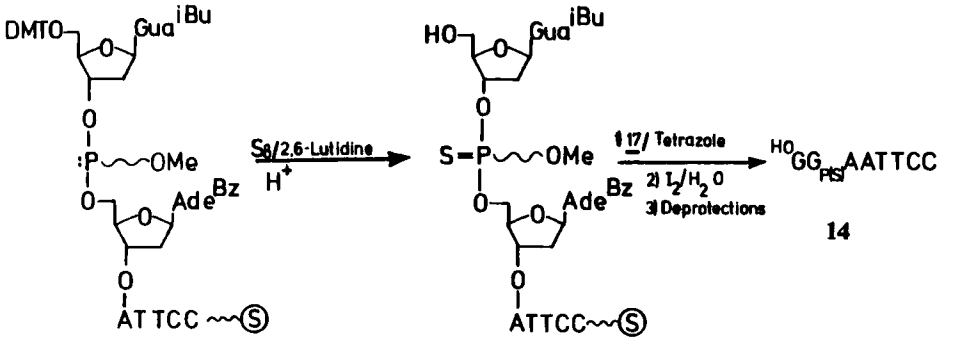
If DN-ase II is responsible for the 3′ → 5′ phosphoryl transfer, the explanation requires to speculate, that capacity of the active center of DN-ase II is limited and the 5′-phosphorylated compound is formed preferably because the rotation of released (Rp)-thymidyl (3′-5′)thymidine phosphorothioate within the cleft of active site is easier than an access of water or another substrate molecule.

Apparently, the fact that primary intermediate **11** does not undergo hydrolysis, which has to be the case for its oxo counterpart, indicates the diversity of phosphorothioate, as compared to phosphate substrates, and also requires speculation about hydrophobicity of enzyme cleft where covalent binding to phosphorus of thymidine 3′-phosphorothioate takes place. All above hypotheses have to be proved or disproved in the future studies, but the observation which we would like to address here, is that 3′ → 5′ 4-nitrophenyl phosphorothioate group transfer, independently on the nature of enzyme which catalyzes this transfer, is stereocontrolled by the 5′-adjacent phosphorothioate center, which is remote from the "scissile" bond through six covalent bonds.

PHOSPHOROTHIOATE ANALOGUES OF OLIGONUCLEOTIDE 5′-GGAATTCC AND THEIR DEGRADATION WITH ENDONUCLEASE Eco RI

The discovery of restriction enzymes had an enormous impact on the development of genetic engineering, but besides practical applications it has raised the question of phenomenal recognition of canonical restriction sequences of DNA by these enzymes and the mechanism of selective cleavage of internucleotide phosphodiester bonds. Among hundreds of restrictases perhaps the most extensive studies on the mechanisms of endonucleolytic action have been performed on Eco RI endonuclease,[24] which recognizes the $^{5'}$ ...GAATTC... complementary sequence on double stranded DNA and causes the cleavage of phosphodiester bond between G and A with formation of ...G$^{-}_{OH}$ and $^{5'}{}_{p}$AATTCC... fragments. It was of interest, whether Eco RI endonuclease-catalyzed cleavage of DNA involves single- or double displacement process, and for this reason phosphorothioate analogue of GGAATTCC octamer (**14**) with phosphorothioate internucleotide bond between G and A was synthesized and separated into diastereoisomeric species. The synthesis of GG$_{P(S)}$AATTCC diastereoisomers (**14**) was achieved by modification of phosphoramidite approach which involves the thioylation of *O*-methylphosphite intermediate **15** by means of 0.4M 2,6-lutidine solution of elemental sulphur followed by deprotection of 5′-hydroxy function and further coupling of CPG-bound heptamer **16** with 5′-dimethoxytrityl N^2-isobutyrylguanosine-3′-*O*-methyl *N*,*N*-diisopropyl phosphoramidite (**17**).

After completion of the last cycle of oligomer synthesis, the routine work-up with thiophenol-triethylamine and concentrated NH_4OH left the product which was isolated and separated into diastereoisomers "fast"-**14** and "slow"-**14** by means of HPLC.[25] The absolute configuration at phosphorus of phosphorothioate moiety was assigned enzymatically. Snake-venom phosphodiesterase catalyzed hydrolysis of "fast"-**14** led to guanosine and seven mononucleotides species, while under the same conditions "slow"-**14** was hydrolyzed with formation of $_{p}$G$_{P(S)}$A, besides other mononucleoside fragments; their treatment with alkaline phosphatase left (Sp)-G$_{P(S)}$A. Independently, both "fast"-**14** and "slow"-**14** were separately hydrolyzed in the presence of nuclease P-1 and it appeared that the digest of "fast"-**14** had contained (Rp)-G$_{P(S)}$A among the other products of hydrolysis. Based on these results, absolute configuration (Rp)- was assigned for "fast"-**14** and (Sp)- for



SCHEME 7

"slow"-**14**. The synthesis and absolute configuration assignments of both diastereoisomers of **14** were simultaneously achieved by Professor Eckstein and from his laboratory the evidence has been reported, that only Rp-**14** is the substrate for Eco RI endonuclease[26] and its hydrolysis occurs with *inversion* of configuration at phosphorus involved in the "scissile" internucleotide phosphorothioate bond.[27] This result clearly indicates that the most probable mechanism for this enzyme does not involve a covalent bond between enzyme acceptor group and phosphorus atom at "scissile" position. Moreover, Eckstein's results indicate the stereoselectivity interaction of this particular phosphate moiety with the binding site of enzyme and the importance of negative charge residing at "scissile" phosphate. However, according to earlier results of Modrich *et al.*[28] from the alkylation interference and protection experiments it became apparent, that besides the "scissile" phosphate ($\downarrow, \nabla$) two other phosphates at adjunct positions towards 5′-end (∇) have to be involved in binding to the enzyme, since their alkylation renders the cleavage of phosphodiester bond between G and A ($\downarrow$).[28]

$$\begin{array}{lll} 5' & A_{\Delta}A_{\Delta}G_{\Delta}^{\downarrow}AATTCTC & 3' \\ 3' & TTCTTAA^{\nabla}_{\uparrow}G^{\nabla}A^{\nabla}G & 5' \end{array}$$

Although these results clearly indicated the importance of the negative charges at adjacent phosphates, it was of interest to check whether an interaction between Eco RI endonuclease and oligonucleotide bearing the sequence GGAATTCC is also diastereoselective with respect to stereogenic phosphate at adjunct position to "scissile" bond. For this reason two oligonucleotides $G_{P(S)}$GAATTCC (**18**) and $G_{P(S)}$GGAATTCCC (**19**) were synthesized and separated into diastereoisomeric species according to methodology described for **14**.[29] Absolute configurations were assigned by means of enzymatic digestions with snake-venom phosphodiesterase (sv PDE) and nuclease P-1. In both diastereoisomeric pairs of compounds **18** and **19** fast-eluted isomers appeared to have (Rp)-configuration (products of digestion with nuclease P-1 gave (Rp)-$G_{P(S)}$G) while slow-eluted ones were of (Sp)-configuration (products of digestion with svPDE did contain (Sp)-$G_{P(S)}$G). Each of isomers of **18** and **19** was individually digested by Eco RI endonuclease (approximately 2 A_{260}

units of each substrate dissolved in a 200 μL of buffer containing 20 mM Tris-HCl, pH 7.6, 160 mM NaCl and 40 mM $MgCl_2$ were incubated with the enzyme, Sigma (400 unites) at 16°C for 24 hours) in parallel with control experiment of digestion of **13** and the reaction progress was followed by means of HPLC. It has been found that after 24 hours of incubation compound **13** was degraded into GG and $_pAATTCC$, while (Sp)-**18** was practically unchanged. Compound (Rp)-**18** was hydrolysed up to 65% into (Rp)-$G_{P(S)}G$ and $_pAATTCC$, while both (Rp)- and (Sp)-**19** were completely degraded to $_pAATTCCC$, (Rp)-$G_{P(S)}GG$ and (Sp)-$G_{P(S)}GG$, respectively.

$$\text{GGAATTCC (13)} \xrightarrow{\text{Eco RI},H_2O} \text{GG} + {}_p\text{AATTCC}$$

$$\text{(Rp)-G}_{P(S)}\text{GAATTCC (18)} \longrightarrow \text{(Rp)-G}_{P(S)}\text{G} + {}_p\text{AATTCC}$$

$$\text{(Sp)-G}_{P(S)}\text{GAATTCC (18)} \not\longrightarrow$$

$$\text{(Rp)-G}_{P(S)}\text{GGAATTCCC (19)} \longrightarrow \text{(Rp)-G}_{P(S)}\text{GG} + {}_p\text{AATTCCC}$$

$$\text{(Sp)-G}_{P(S)}\text{GGAATTCCC (19)} \longrightarrow \text{(Sp)-G}_{P(S)}\text{GG} + {}_p\text{AATTCCC}$$

The results obtained with **18** fully confirmed the conclusion of Modrich *et al.*[28] that the negative charge at phosphate of 5′-adjacent position to "scissile" phosphate is essential for interaction of DNA with Eco RI endonuclease.

Moreover, observed results indicate that most probably an oxygen of the pro-S phosphate at this position is involved in binding with positively charged functions of Eco RI endonuclease. This interaction is diastereoselective and consists of an example of "remote" control of Eco RI endonuclease-catalyzed cleavage at "scissile" site of DNA bearing canonical sequence ...GAATTC... by the stereochemistry of "adjacent" 5′-phosphorothioate.

It should be pointed out that 5′-"adjacent" phosphorothioate group is separated from cleavable bond through six covalent bonds. The lack of selectivity of endonuclease Eco RI towards **19** may indicate that internucleotide phosphate separated by two deoxyribose moieties from "scissile" phosphate is not involved in the binding to the enzyme, but this conclusion must be verified by means of experiment with diastereoisomers of $G_{P(O)OEt}GGAATTCCC$.[30]

DISCUSSION

The results obtained on DN-ase II catalyzed "hydrolysis" of diastereoisomers of compound **2** have demonstrated that DN-ase II, similarly to spleen phosphodiesterase,[31] exerts the transferase activity, and the chain elongation occurs with retention of configuration, which speaks for involvement of thymidyl 3′-phosphorothioylated enzyme **11** as an active intermediate. However, the product (Rp,Sp)-**3** undergoes further transformation via intermediacy of 4-nitrophenyl phosphorothioylated enzyme **12** which in reaction with (Rp)-thymidyl (3′-5′)thymidine phosphorothioate gives (Sp,Rp)-**4**. This complicated, and to some extent confusing result, may consist of the first example of dual activity of this nuclease, although the

possibility, that 3′ → 5′4-nitrophenyl phosphorothioate transfer is caused by another protein contaminating DN-ase II, has to be considered and requires experimental check. The different behaviour of phosphorothioates, as compared with phosphates, also awaits further elucidation, but the hypothesis is formulated that observed differences are caused by different hydrophobicity of these two types of substrates.

An observation that only (Rp,Sp)-diastereoisomer of **3** undergoes 3′ → 5′ 4-nitrophenyl phosphorothioate transfer consist of an example of "remote" control of diastereoselectivity of enzymatic process. The same type of "remote" control of enzymatic reaction is observed for hydrolytic activity of Eco RI endonuclease which accepts as the substrate (Rp)-**18** only. In this particular case the number of factors can be considered, since the lack of activity of Eco RI endonuclease towards (Sp)-**18** may indicate the conformational changes of **18** caused by the presence of phosphorothioate moiety at internucleotide 5′-"adjacent" position to "scissile" bond, which hampers the recognition process. An alternative explanation may be offered, that catalytic action of protein can not be executed without strong interaction between positively charged group of the enzyme and negatively charged phosphate at 5′-"adjacent" position to "scissile" bond. If the charge distribution within phosphorothioate anion is not symmetrical[32] and negative charge is located on the oxygen atom, then isomer of **18** which possess the phosphorothioate orientated with its oxygen "outside" the enzyme can not be involved in the close enough contact with protein molecule. The necessary DNA-protein interface is not achievable, because the rotation of phosphorothioate to gain the proper alignment of phosphate back-bone disturbs the recognizable arrangement of base within canonical sequence ...GAATTC... .

ACKNOWLEDGMENT

This work was financially assisted by the Polish Academy of Sciences, grant No MR-I-12. Authors are indebted to Professor Gerald Zon, FDA, Bethesda, MD, USA, for his enthusiastic participation in the initial studies on Eco RI endonuclease-DNA interactions.

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