Inhibition of deoxyribonucleases by phosphorothioate groups in oligodeoxyribonucleotides

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ABSTRACT
The Rp- and Sp-diastereomers of the phosphorothioate-containing oligonucleotide d[ApAp(S)ApA] have been synthesized. They and the tetramer d[ApApApA] were tested as substrates for staphylococcal nuclease, DNase II and spleen phosphodiesterase. For digestions with DNase I these oligonucleotides were converted to the 5'-phosphorylated derivatives. The reactions with the nucleases were analysed by HPLC. The phosphorothioate groups of both diastereomers were resistant to the action of staphylococcal nuclease, DNase I and DNase II. While the phosphorothioate group of the Rp-diastereomer was resistant to the action of spleen phosphodiesterase, the Sp-diastereomer was hydrolysed at an estimated rate 1/100 the rate of cleavage of the unmodified tetramer. The presence of the phosphorothioate group in the center of the molecule affected the rate of hydrolysis of neighbouring phosphate groups for some enzymes. In particular, very slow release of 3'-dAMP from the Rp-diastereomer occurred on incubation with staphylococcal nuclease but the Sp-diastereomer was completely resistant. DNase II produced 3'-dAMP quite rapidly from both diastereomers of d[ApAp(S)ApA] and DNase I released 5'-dAMP from both diastereomers of d[pApAp(S)ApA] only slowly.

INTRODUCTION
The presence of phosphorothioate groups in oligodeoxynucleotides, polynucleotides, DNA and RNA often stabilizes such compounds against degradation by nucleases (1). The reduction of the rate of degradation of such phosphorothioate-containing nucleic acids depends on the particular nuclease employed. In many cases such modified compounds are virtually resistant to the action of a given nuclease whereas in others only a small effect can be observed. Examples are the protection against the action of 3'-5'-exonuclease (2, 3), and 5'-3'-exonuclease of DNA polymerase I (4-7), exonuclease III (8), certain restriction endonucleases (9,10), topoisomerases (11), E. coli integration host factor (12), snake venom phosphodiesterase (5,13), nucleases S1 and P1 (14,15), RNases (16) and serum nucleases (16,17). However, some of the more commonly used deoxyribonucleases have not yet been studied systematically, although there is a report which describes qualitatively a decrease in the rate of breakdown of DNA by
DNase I in the presence of phosphorothioate groups (18). No investigation of the degradation of phosphorothioate-containing oligonucleotides by staphylococcal nuclease, pancreatic DNase I, hog spleen DNase II and spleen phosphodiesterase has yet been reported. We have chosen to compare the action of these enzymes on the oligodeoxynucleotide d[ApApApA] and the two diastereomers of its phosphorothioate analogue d[ApAp(S)ApA] in order to obtain information on the ability of phosphorothioate groups to protect internucleotidic linkages against the action of these nucleases. Tetramers are the shortest oligonucleotides with which endonucleolytic as well as exonucleolytic activities of nucleases can be studied. As we were most interested in studying the effect of phosphorothioates towards endonucleolytic activities we placed this group in the center of the tetramer.

EXPERIMENTAL
Materials and Methods
Chemicals: 5-(p-Nitrophenyl)-tetrazole was synthesized by the procedure of Finnegan et al. (19), methoxy-morpholino-chlorophosphine as described by McBride and Caruthers (20), N6-benzoyl-3'-O-methoxyacetyl-2'-deoxyadenosine by the procedure of Potter and Eckstein (21), N6-benzoyl-5'-O-dimethoxytrityl-2'-deoxyadenosine by the procedure of Gait et al. (22). The latter was reacted with methoxy-N,N-diisopropylamino-chloro-phosphine as described by Dörper and Winnacker (23) to the 3'-phosphoroamidite. This compound and the phosphoroamidite 4 were purified by flash chromatography on silica-gel with a mixture of CH2Cl2/EtOAc/Et3N (45/45/10, v/v) and judged to be pure by 31P-NMR-spectroscopy. The pure deoxynucleoside-3'-O-phosphoramidites were precipitated into cold hexane and stored at -20°C. Oligo-p(dA)4 and oligo-(dA)4 were purchased from Pharmacia P-L Biochemicals (Piscataway, USA) as the lyophilized sodium or ammonium salts. 5'-dAMPS was prepared according to Murray and Atkinson (24). A molar extinction coefficient of 60.000 was used for all tetramers.

NMR-Spectra: 31P-NMR-spectra were recorded on a Bruker WP 200 SY spectrometer operating at a frequency of 81.01 MHz with quadrature detection and 1H broad-band decoupling with 85% aqueous phosphoric acid as external standard. Chemical shifts are given in parts per million (ppm) and are positive downfield from the standard. Samples which were soluble in organic solvents were recorded in CDCl3, whereas spectra of samples soluble in aqueous media were measured in 100 mM TRIS (pH 8.0) and 100 mM EDTA containing 25% D2O.

HPLC analysis: Reverse phase HPLC on ODS Hypersil (Shandon Southern Prod., Cheshire, UK) was performed with two Waters Associates Model 6000A pumps.
controlled by a Model 660 solvent programmer. The mobile phase was 100 mM triethylammonium bicarbonate (pH 7.5), with a linear gradient of acetonitrile from 0 to 30% in 15 min. The flow-rate for these analytical runs was 1.5 ml/min.

**Enzymes** : The following enzymes were purchased from Boehringer (Mannheim, FRG) : Deoxyribonuclease I from bovine pancreas grade I (4,000 units/ mg; a total of 20,000 units was dissolved in 2 ml H2O); endonuclease from staphylococcus aureus (15,000 units/mg; 1 mg was dissolved in 1 ml H2O); nuclease P1 from penicillium citrinum (300 units/mg; 1 mg was dissolved in 300 µl H2O); alkaline phosphatase from calf intestine, grade I (1700 units/mg; 7,500 u were suspended in 890 µl ammonium-sulfate solution, pH 7); and phosphodiesterase from calf spleen (2 units/mg; 4 units in 1 ml ammonium-sulfate suspension, pH 6). Deoxyribonuclease II from porcine spleen (14,540 units/mg, a total of 20,000 units was dissolved in 2 ml H2O) was obtained from SIGMA Chemical Company (St. Louis, USA), and T4 polynucleotide kinase (2,500 units/100 g) was supplied by US-Biochemicals (Cleveland, USA) as a solution in 50% glycerol, containing 50 mM TRIS-HCl (pH 7.5), 1.0 mM DTT and 0.1 mM EDTA. For digestions with these enzymes the following buffers were used : DNase I, buffer I : 100 mM TRIS-HCl (pH 7.4), 10 mM CaCl2 and 10 mM MgSO4 ; DNase II, buffer II : 50 mM sodium-acetate (pH 5.0); staphylococcal nuclease, buffer III : 100 mM TRIS-HCl (pH 7.4), 5 mM CaCl2 ; nuclease P 1, buffer IV : 100 mM TRIS-HCl (pH 7.8), 5 mM MgCl2 ; alkaline phosphatase, buffer V : 100 mM triethylammonium bicarbonate (pH 7.5); and calf spleen phosphodiesterase, buffer VI : 100 mM sodium-citrate (pH 6.0).

**Chemical Synthesis**

The synthetic route for the phosphorothioate oligonucleotide 6 is outlined in scheme 1a and b. The fully protected tetradeoxyadenosyl-phosphorothioate 5 was synthesized essentially by the method of Matteuci and Caruthers (25) by block-condensation in solution. After synthesis of 1 the 3’-O-methoxyacetyl protecting group was removed from approximately one half of the material (500 µmol) by a short treatment with a mixture of aqueous ammonia and dioxan. The resulting deprotected dimer 3 was phosphitylated with methoxy-N,N-diisopropyl-amino-monochlorophosphine to give the 3’-O-phosphitylated dinucleotide 4. The other half of the fully protected dinucleotide 1 was detritylated to the dimer 2 and coupled with 4 upon activation with 5-nitrophenyl-tetrazole (26). The reaction product was dissolved in acetonitrile and reacted with sulfur (four-fold excess), dissolved in a solution of pyridine and carbon disulfide (2:1,v/v), for 30 min. at room temp.(4). The fully protected tetramer was obtained after chromatography on silica gel with a linear gradient of chloroform containing 2-8% methanol as a mixture of eight diastereomers. 31P-NMR-spectroscopy in CDCl3 revealed the presence of one
phosphorothioate triester with signals at around 69 ppm (δ = 69.92, 69.87, 69.83, 69.79, 69.76 and 69.74 ppm) and two phosphate triesters with signals between 0.01 and -0.54 ppm (δ = 0.01, -0.43, 0.99, -0.11, -0.28, -0.49, -0.52 and -0.54 ppm) in the expected ratio of 1:2. The protecting groups were removed by treatment with acetic acid, followed by reaction with thiophenolate and incubation with ammonia overnight. This reduces the number of diastereomers to two. The crude oligonucleotide phosphorothioate 6 was purified by chromatography on a DEAE A-25 Sephadex-column with a linear gradient of triethyl ammonium bicarbonate. 31P-NMR-spectroscopy of the purified product clearly revealed the presence of the two diastereomers of the phosphorothioate at δ = 55.53 and 55.06 ppm in a ratio of 1.2:1.0. The mixture of the two diastereomers was resolved by preparative HPLC on a RP-column with triethylammonium bicarbonate (pH 7.5) containing increasing concentrations of acetonitrile from 0 to 30% in 30 min. Retention times were 26.1 min
(isomer A) and 27.6 min (isomer B). The purity of each diastereomer was greater than 98%.

Both diastereomers of 6 were digested with nuclease P1 for assignment of their absolute configuration. This enzyme hydrolyses stereospecifically a dinucleoside phosphorothioate with the Sp-configuration (14). Digestion of isomer A gave three products in a ratio of 1:1:2. Two were identified as 5'-dAMP and dA. After short treatment with alkaline phosphatase, the third product was shown to be the Rp-isomer of d[Ap(S)A] through comparison with an authentic sample prepared by the method of Burgers and Eckstein (5). This proved that isomer A has the Rp-configuration at the phosphorothioate position. Digestion of isomer B with nuclease P1 gave 5'-dAMP, 5'-dAMPS and dA in a ratio of 2:1:1. Since the phosphorothioate-group of this diastereomer was completely digested, it was assigned to be the diastereomer with the Sp-configuration. This result follows the empirical rule that the isomer of an oligonucleotide-phosphorothioate with the shorter retention time in reverse-phase HPLC is the Rp-diastereomer, whereas the isomer with the longer retention time is the Sp-diastereomer (1).

Each individual diastereomer was phosphorylated with polynucleotide kinase and ATP to give the phosphorylated derivates d[pAp(S)ApA] and purified by semi-preparative HPLC.

**Enzymatic Digestions**

*Digestion of oligo-(dA)₄ by staphylococcal nuclease*: It was first established, by determining the rate of hydrolysis as a function of substrate concentration, that the enzyme was saturated at around 10 μM oligonucleotide. For the identification of reaction products, oligo-(dA)₄ (0.5 A₂₆₀-unit, 8.4 nmol) was incubated with 5.0 units of staphylococcal nuclease in a total volume of 100 μl of buffer III for 4 hours at 37°C. The sample, as analysed by HPLC, was completely digested to four new products with retention times of 6.0, 6.8, 7.1 and 7.8 min in a ratio of approximately 1:1:3:1. Three were identified as 3'-dAMP (retention time 6.0 min), dA (6.8 min) and d[ApA] (7.8 min). For the analysis of the fourth product, the sample was further incubated with 2 μl of alkaline phosphatase solution for 5 min at 37°C. HPLC analysis showed only two products, dA and d[ApA], with retention times of 7.0 and 7.8 min in a ratio of 1:2. Thus, the fourth product of the first digest must have been d[ApAp] (retention time 7.1 min). The formation of products as a function of time is shown in fig. 1.

*Digestion of Rp- and Sp-d[ApAp(S)ApA] (6) by staphylococcal nuclease*: Each diastereomer (17 nmol) of d[ApAp(S)ApA] was incubated with 20 units of staphylococcal nuclease for 2 h at 37°C in a total volume of 200 μl of buffer III and
Figure 1: Kinetic course of the digestion of oligo-(dA)₄ by Staphylococcal nuclease. Oligo-(dA)₄ (8.4 nmol) was incubated with 0.5 unit of enzyme in 100 µl of buffer III at 37°C. Aliquots of 10 µl were removed at times indicated and analysed by HPLC Oligo-(dA)₄, (∣); d[ApAp], (■) d[ApA], (○) 3'-dAMP and dA (○).

then analysed by HPLC. The Rp-isomer was completely digested to dA (retention time 7.0 min) and another product (7.7 min) in a ratio of 1.0 : 2.6. For the analysis of the second product, the sample was first treated with 2 units nuclease P1-solution for 30 min and then with 2 µl of aqueous alkaline phosphatase solution for 60 min at 37°C. HPLC analysis showed that upon addition of nuclease P 1 the amount of dA increased, while the intensity of the second product was decreased. Upon incubation with alkaline phosphatase this product was dephosphorylated to a new compound, retention time 8.7 min, which was identical with the Rp-isomer of d[Ap(S)A]. Thus, the second product of the initial reaction must have been Rp- d[ApAp(S)Ap]. The Sp-isomer was only degraded to an extent of 10%. The distribution of products as a function of time is shown in fig. 2.

_Digestion of oligo-p(dA)₄ by deoxyribonuclease I_: It was first established that the enzyme was saturated at a concentration of approximately 80 µM of oligo-p(dA)₄. For the identification of products oligo-p(dA)₄ (8.4 nmol) was incubated with 2 µl (20 units) DNase I-solution in a total volume of 100 µl of buffer I at 37°C. After 24 h HPLC analysis showed complete conversion of the starting-material to one product (retention time 6.4 min). Since this product could not be directly assigned, 5 µl of alkaline phosphatase solution was added to the reaction mixture and incubated for 5 min at 37°C. The unknown product was converted to the dinucleoside d[ApA]
Figure 2: Kinetic course of the digestion of d\([ApAp(S)ApA]\) by Staphylococcal nuclease. Rp- or Sp-d\([ApAp(S)ApA]\) (17 nmol) was incubated with 10 units of enzyme in 200 μl of buffer III at 37°C. Aliquots of 10 μl were removed at times indicated and analysed by HPLC. Sp-isomer, (○); Rp-isomer, (□); Rp-isomer of d\([ApAp(S)ApA]\), (■); dA, (●).

(retention time 7.4 min). Thus, the product of the DNase I digest must have been d[pApA]. The time course of product formation is shown in fig. 3.

Digestion of Rp- and Sp-isomer of d[pApAp(S)ApA] by deoxyribonuclease I: Both diastereomers of d[pApAp(S)ApA] (8.4 nmol) were incubated with 10 μl (100 units) of DNase I-solution in a total volume of 100 μl of buffer I. After incubation for 24 hours at 37°C both samples were analysed by HPLC. Both diastereomers were digested to two new products with retention times of 7.2 and 7.3 min in a ratio of approximately 1 : 2. Since one of these products could be identified as dA (retention time 7.2 min), the other was assigned as the diastereomer of d[pAp(S)ApA].

Digestion of oligo-(dA)₄ by calf-spleen phosphodiesterase: Oligo-(dA)₄ (17 nmol) were incubated with 5 μl of calf-spleen phosphodiesterase suspension in a total volume of 100 μl buffer-solution VI for 1 hour at 37°C. HPLC analysis showed complete conversion of the starting material (retention time 8.1 min) to two new products with retention times of 5.7 and 6.1 min in a ratio of 1 : 3. The minor product was identical with deoxyinosine (retention time 5.7 min), obtained by deamination of dA, while the major product of the enzymatic digestion was 3'-dAMP (retention time 6.1 min).

Digestion of the Rp- and Sp-isomer of d[ApAp(S)ApA] by calf-spleen phosphodiesterase: Each isomer of d[ApAp(S)ApA] (33 nmol) was incubated with 20 μl of
Figure 3: Kinetic course of the digestion of oligo-p(dA)₄ by DNase I. Oligo-p(dA)₄ (8.4 nmol) was incubated with 20 units of enzyme in 100 µl of buffer I at 37°C. Aliquots of 10 µl were removed at times indicated and analysed by HPLC. Oligo-p(dA)₄, (○); d[pApA], (●).

calf-spleen phosphodiesterase suspension in a total volume of 200 µl of buffer VI at 37°C. After 1 hour, HPLC analysis showed that both diastereomers had been digested to 3’-dAMP, (retention time 6.0 min) and d[Ap(S)ApA] (Rp-isomer, retention time 8.6 min, and Sp-isomer, retention time 9.1 min). After digestion for 120 hours, 90% of the Sp-isomer was hydrolysed to dA which, however, had been deaminated to dl (retention time 5.6 min), 3’-dAMP (retention time 6.1 min) and a new product with the retention time of 6.6 min in a ratio of approximately 1:2:1. While the first two products could be identified by coinjection with authentic samples, the third product was assigned as 3’-dAMPS (retention time 6.6 min). The Rp-isomer, however, was hydrolysed by approximately 60% in 120 h yielding mainly Rp-d[Ap(S)Ap].

Digestion of oligo-(dA)₄ by deoxyribonuclease II : Oligo-(dA)₄ (8.4 nmol) was incubated with 5 µl (5 units) of DNase II-solution in a total volume of 100 µl of buffer II for 18 hours at 37°C and analysed by HPLC. After incubation for 60 min, the oligonucleotide (retention time 8.0 min) was first digested to a new compound (retention time 7.5 min) which we identified as the trimer d[ApApA] and 3’-dAMP (retention time 5.3 min). Upon further incubation, this mononucleotide was slowly dephosphorylated to dA (retention time 6.7 min), while the remaining trimer was further degraded to 3’-dAMP and dA. Finally, a 3 : 1 mixture of these products was obtained.
Digestion of the Rp- and Sp-isomer of d[ApAp(S)ApA] (6) by deoxyribonuclease II:
Each isomer of the two oligonucleotide-phosphorothioates 6 (17 nmol) was incubated with 10 μl (100 units) of DNase II-solution in a total volume of 200 μl of buffer II for a period of 120 h at 37°C. The samples were checked by HPLC after appropriate intervals. After 2 h incubation the two diastereomers were digested to 3'-dAMP (retention time 5.7 min) and the diastereomers of d[Ap(S)ApA] (Rp-isomer, retention time 8.5 min, and Sp-isomer, retention time 9.1 min). After incubation for 18 hours, the mononucleotide was completely dephosphorylated to dA (retention time 6.8 min). Upon further digestion, the Rp-isomer was finally degraded to dA and d[Ap(S)Ap] (retention time 8.7 min) which was identified by conversion into Rp-d[Ap(S)A] (retention time 9.1 min) by a short treatment with 2 μl alkaline phosphatase for 5 min. The Sp-d[Ap(S)ApA] was further digested to some unidentified product which was presumably Sp-d[Ap(S)Ap].

RESULTS AND DISCUSSION
The interest in phosphorothioate analogues of nucleotides basically rests on two characteristics of these compounds. One is their suitability for the elucidation of the stereochemical course of enzyme catalysed phosphoryl and nucleotidyl transfer reactions (1, 27, 28, 29) and the other is the inability of many hydrolysing enzymes to efficiently cleave phosphorothioate esters (1). This latter property has aroused much interest, in particular with respect to the protection of the phosphodiester internucleotidic linkage against enzymatic cleavage by the incorporation of phosphorothioate groups into oligonucleotides or DNA. Some examples of this effect are listed in the introduction.

We were interested in extending such studies to DNases such as staphylococcal nuclease and DNase I for which the effect of phosphorothioate groups on the cleavage of oligonucleotides, as models for DNA, has not yet been determined. We first considered d[ApApTp] as a suitable oligonucleotide for such an investigation as it had been described as a substrate for DNase I (30). However, we were unable to reproduce this result. The oligonucleotide became a substrate only after 5'-phosphorylation and was then cleaved to 5'-dAMP and the 3'-phosphorylated dinucleotide d[pApTp], as well as to thymidine-5',3'-bisphosphate and the dinucleotide d[pApT] (Spitzer and Eckstein, unpublished results). Another report describes the endonucleolytic cleavage of the two tetramers d[pApApApA] and d[pTpTpTpT] by DNase I into the corresponding dinucleotide 5'-phosphates (31). We considered such tetramers as more suitable molecules for our study as they are cleaved only at one internucleotidic linkage. Replacement of this by a phosphorothioate group should then quite readily reveal the effect of this group. We
therefore prepared d[ApAp(S)ApA], the phosphorothioate analogue of d[ApApApA], as a mixture of diastereomers by block condensation of two partially protected dimers which were separated and used as substrates for the DNases. A schematic representation of the products obtained in the various nuclease reaction is given in scheme 2.

**Staphylococcal nuclease**

The nuclease from *staphylococcus aureus* is a phosphodiesterase which has been extensively studied (for a review, see ref. 32). It cleaves single-stranded DNA more rapidly than double-stranded DNA. Ho and Gilham (33) report that the tetramers d[pApTpApT] and d[pTpApTpA] are digested by the enzyme both in an endo- and exonucleolytic manner. The endonucleolytic cleavage preferentially occurred at a dNp-dA-bond. The oligonucleotides so formed were then degraded by the exonuclease starting from the 3'-end resulting in the release of mononucleosides and nucleotides. Accordingly, we find that the nuclease degrades d[ApApApA] to 3'-dAMP, dA, d[ApAp] and d[ApA] in a ratio of 1:1:3:1. These are the expected products of the two competing activities, the endo- as well as the exonucleolytic activity. The former cleaves the tetramer in the centre to yield d[ApAp] and d[ApA], the latter degrades it from the 3'-end to first produce dA and d[ApApAp] which is further cleaved to d[ApAp] and 3'-dAMP. The ratio of the final products which allows an estimate of the relative velocities of the two nucleolytic activities because the dimers are not substrates for further nucleolytic action, indicates that the exonuclease activity is twice as fast as the endonuclease activity. Of the two diastereomers of d[ApAp(S)ApA] only the Rp-diastereomer is cleaved. However, the rate is 10 times slower than for the unmodified tetramer and only the first step of the exonucleolytic activity producing dA and Rp-d[ApAp(S)Ap] is observed. Neither Sp-d[ApAp(S)ApA] nor Rp-d[ApAp(S)Ap] are cleaved thus showing that the endonucleolytic activity is completely inhibited by the phosphorothioate group.

These results demonstrate not only a long range effect caused by the phosphorothioate group on the cleavage of a neighbouring phosphodieste group in the 5'-position but also the differential effect of the two diastereomers. The influence of neighbouring internucleotidic groups on each other has also been observed for other nucleases, the best example probably being that of the restriction enzyme Nci I (34). The presence of a phosphorothioate group solely at the position of cleavage is not sufficient to prevent cleavage by this enzyme but an additional phosphorothioate group in the neighbouring 3'-position prevents hydrolysis. A similar effect has been reported for the restriction endonuclease Eco RI (35) and has been observed for many others (Olsen, Sayers, Eckstein unpublished).
Scheme 2: Hydrolysis of oligo-(dA)₄ and the diastereomers of d[ApAp(S)ApA] by the various nucleases.

**Pancreatic DNase I**

Pancreatic deoxyribonuclease degrades double-stranded DNA endonucleolytically into oligonucleotides of an average chain length of 4 nucleotides with a free hydroxyl group at the 3'- and a phosphate group at the 5'-position (36). In agreement with the results obtained by Ralph et al. (30) we find that d[pApApApA] was cleaved in the center of the molecule to yield a single product, d[pApA]. The half-life of the digestion of the tetramer was approximately 6 h when 20 units of enzyme were employed. Under these conditions no cleavage of either the phosphorylated Rp- or Sp-diastereomer of the phosphorothioate analogue of the tetramer was observed. However, when the digestion of both diastereomers was repeated with a 5-fold larger amount of DNase I, the enzyme degraded both tetramers at the phosphodiester bond which is adjacent to the phosphorothioate group in the 5'-direction. Thus, 5'-dAMP which was subsequently dephosphorylated was released from both...
samples and a chiral trinucleotide phosphorothioate was the final product. Thus, the phosphorothioate groups in both diastereomers were not hydrolysed.

**Spleen phosphodiesterase**

This enzyme is an exonuclease and degrades oligonucleotides in a sequential manner starting at the 5'-terminus to nucleoside 3'-monophosphates. The enzyme is active on both RNA and DNA (37). The substrate specificity of calf spleen phosphodiesterase towards phosphorothioates has been studied with the diastereomers of thymidine 3'-O-(4-nitrophenyl) phosphorothioate. The enzyme catalysed transnucleotidylation rather than hydrolysis of both diastereomers without any significant stereoselectivity towards one isomer of the chiral phosphorothioate (38). The mixture of diastereomers of the dinucleoside phosphorothioate [Tp(S)T] was shown earlier (39) not to be a substrate for this enzyme but a competitive inhibitor. The enzyme readily cleaves d[ApApApA] to 3'-dAMP and dA in a ratio of 3:1. Both the Rp as well as the Sp-diastereomer of d[ApAp(S)ApA] are cleaved quite fast in a first step to 3'-dAMP and Rp- and Sp-d[Ap(S)ApA], respectively. The Sp-diastereomer of this intermediate is further hydrolyzed slowly to 3'-dAMP, 3'-dAMPS and dA. The course of further hydrolysis for the Rp - diastereomer of the intermediate proceeded somewhat differently in that small amounts of Rp-d[Ap(S)Ap] were generated. The degradation of the unmodified tetramer is estimated to be approximately 100 times faster than that of the phosphorothioate group in the Sp-isomer. We did not observe any products derived from a nucleotidyl transfer reaction as seen by Mehdi and Gerlt (40) and Niewiarowski and Uznanski (38) in the reaction with thymidine 3'-[4-nitrophenyl]-phosphorothioate. These results show that spleen phosphodiesterase can cleave the phosphorothioate internucleotidic linkage only of the Sp-isomer and that this cleavage is very slow.

**Hog spleen DNase II**

Double- and single-stranded DNA is degraded by hog spleen DNase II, the latter being the poorer substrate, to oligonucleotides of a chain length between 6 and 14 nucleotides with a phosphate group at the 3'-position and a free 5'-hydroxyl function (41). The stereospecificity of the enzyme towards phosphorothioate groups in oligonucleotides has been reported for the reaction with the diastereoisomers of thymidine 3'-O-(4-nitrophenyl) phosphorothioate (42). It was found that the Rp-diastereomer readily underwent a transnucleotidylation reaction to a dinucleotide 3'-O-(4-nitrophenyl) phosphorothioate.

We find that DNase II degrades d[ApApApA] rapidly to 3'-dAMP and dA in a ratio of 3:1. The half-life for this digestion was 2.5 hours when 5 units of enzyme were used. However, upon prolonged incubation with the enzyme the mononucleotide is
dephosphorylated presumably by contaminating phosphatases. Both the Rp- as well as the Sp-diastereomer undergo the first cleavage step to 3'-dAMP and the corresponding isomer of the trinucleotide-phosphorothioate d[Ap(S)ApA] quite fast. The second step which produces dA by attack from the 3'-end of this trimer is very slow. The approximate difference in rate of this cleavage step to that of the normal tetramer is 100-fold. No cleavage of the phosphorothioate group of either configuration is observed. There is no evidence for products derived from a nucleotidyl transfer reaction as reported for the reaction with thymidine 3'-(p-nitrophenyl) phosphorothioate (42).

CONCLUSION
The study reported here shows that staphylococcal nuclease, DNase I and II do not hydrolyse phosphorothioate internucleotidic linkages of either configuration. Spleen phosphodiesterase hydrolyses a phosphorothioate group of the Sp-configuration with 1/100 the rate of an unmodified phosphate group. The presence of the phosphorothioate group reduces the rate of hydrolysis of neighbouring phosphate groups catalysed by staphylococcal nuclease and DNase I. These results suggest that the introduction of phosphorothioate groups into oligonucleotides and DNA will provide protection against hydrolysis by the enzymes discussed in this publication.

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