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Synthesis of phosphorothioate-containing DNA fragments by a modified hydroxybenzotriazole phosphotriester approach

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ABSTRACT

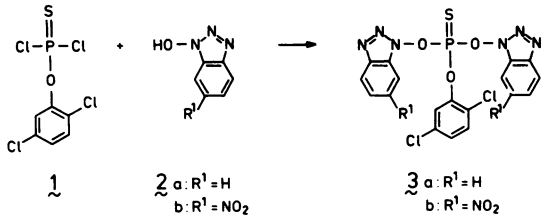
The phosphorothioylating agent which was obtained by treating 2,5-dichlorophenyl phosphorodichloridothioate with 1-hydroxy-6-nitrobenzotriazole proved to be very effective for the synthesis in solution and on a solid support of phosphorothioate-containing DNA fragments.

INTRODUCTION

It is well established, mainly due to the pioneering work of Eckstein et al.[1], that nucleic acids which contain at a specific location a chiral phosphorothioate, instead of the naturally occurring phosphodiester linkage, are valuable compounds to study the stereochemical course of phosphorylitic enzymes. As part of a programme to synthesize phosphorothioate-containing DNA fragments which may serve as probes for molecular biological studies[2], and, further, as model compounds to deepen our insight into the conformational behaviour of Z-DNA[3], we explored the phosphotriester approaches so far developed for the preparation of this type of molecules.

The synthesis of a RNA dimer having a phosphorothioate linkage was achieved for the first time by Burgers and Eckstein[4,5]. Unfortunately, the final unblocking of the intermediate phosphorothioate triester linkage was not completely selective. A more successful approach to the synthesis of phosphorothioate-containing nucleic acids consisted of the introduction of an intermediate phosphite triester, which was subsequently converted by treatment with sulfur into the required phosphorothioate function[6-10].

Recently, Kemal et al.[11] showed that the reaction of the bifunctional phosphorylating agent 2,5-dichlorophenyl phosphorodichloridothioate (1 in Scheme 1) with 1-hydroxybenzotriazole (2a; R^1=H) affords the activated agent (3a; R^1=H), which could be used for the introduction of an intermediate phosphorothioate triester linkage the protective group of which could be removed selectively and stereospecifically. The rate of formation, however, of the



1 2 a: R^1 = H; b: R^1 = NO_2 3 a: R^1 = H; b: R^1 = NO_2

Scheme 1

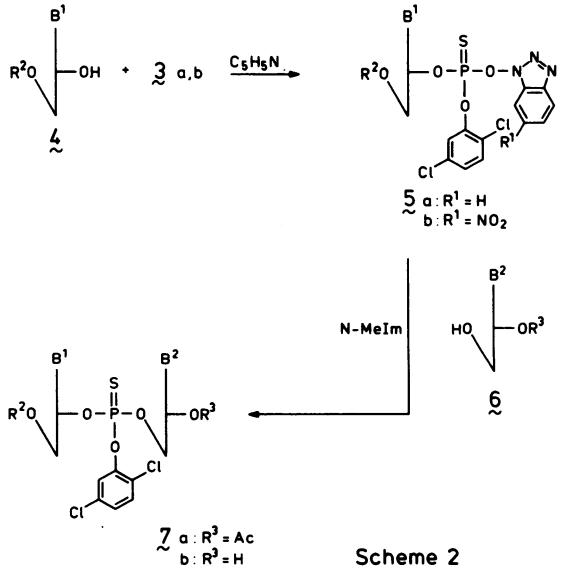
required phosphorothioate linkage (e.g. formation of 7a in Scheme 2) was rather slow.

We report that the conversion of agent 1 with 1-hydroxy-6-nitrobenzotriazole[12] leads to the formation of a more reactive phosphorothioylating agent (i.e. 3b; R^1=NO_2) which can be used successfully for the synthesis in solution and on a solid support of phosphorothioate-containing DNA fragments.

RESULTS AND DISCUSSION

Synthesis of phosphorothioate-containing DNA fragments in solution.

As mentioned previously, the two-step formation of the required 3'-5'-phosphorothioate triester linkage by using agent 3a (R^1=H), as developed by Kemal et al.[11], is rather sluggish. Thus, the total time required to convert 4 (R^2=pixyl; B^1=T, see Scheme 2) into 5a (R^1=H) followed by the addition of



5 a: R^1 = H; b: R^1 = NO_2 7 a: R^3 = Ac; b: R^3 = H

Scheme 2

the 3'-protected d-nucleoside 6 (B^2=T; R^3=acetyl), to afford 7a, was 6.7 h. We reasoned that the reactivity of the phosphorothioylating agent 1 could be increased by replacing the chlorine atoms by benzotriazolyl functions having better leaving capacities than the 1-benzotriazoloxy groups. Measurements of the pK values of 1-hydroxy-6-nitrobenzotriazole (2b; R^1=NO_2) and 1-hydroxybenzotriazole (2a; R^1=H) revealed that 2b was, as expected, more acidic (difference of roughly one pK value) than 2a. In order to find out if 3b (R^1=NO_2), which was obtained by adding 2b to 1 in the presence of pyridine, was indeed an effective phosphorylating agent, we treated the 5'-protected d-nucleoside 4 (B^1=T; R^2=dimethoxytrityl) with a slight excess of 3b (R^1=NO_2) in the presence of pyridine. T.l.c.-analysis, after 15 min, showed the absence of 4 and the presence of a product with zero mobility (i.e. 5; R^1=NO_2, see also Experimental part). A slight excess of the d-nucleoside 6 (B^2=A^{dpa}; R^3=H), having the 3'- and 5'-OH groups free, was added together with N-methylimidazole (N-MeIm). T.l.c.-analysis of the reaction mixture, after 1 h at 20°C, showed, apart from the presence of a small quantity of 6, a new product together with some baseline material. Work-up of the mixture, followed by a rapid column purification step, afforded a mixture containing the higher and lower R_f-diastereoisomers of 7b (B^1=T; B^2=A^{dpa}; R^2=DMTr; R^3=H). The two isomers were separated by short column chromatography to give the higher R_f [R_f 0.39; δ_p ($CDCl_3$) 62.3 p.p.m.] and lower R_f [R_f 0.30; δ_p ($CDCl_3$) 62.0 p.p.m.] diastereoisomers of 7b in isolated yields of 46% and 13% [based on 4 (B^1=T; R^2=DMTr)], respectively. The individual diastereoisomers were now deblocked as follows. Acetylation of 7b gave the corresponding 7a (R^3=Ac) derivatives. The stereospecific removal of the phosphorothioate protecting group was effected by treating 7a with 2-pyridinealdoxime and N^1,N^1,N^3,N^3-tetramethylguanidine[13] in dry acetonitrile. The acetyl (R^3) and diphenyl acetyl (dpa) groups were then removed by aqueous ammonia. Finally, removal of the DMTr-group (R^2) with aqueous acetic acid, and purification of the products by anion-exchange chromatography, afforded the chirally pure diastereoisomers of d[Tp(s)A]. ^{31}P-NMR of the fully-deblocked diastereoisomer derived from the higher R_f-diastereoisomer of 7b (B^1=T; B^2=A^{dpa}; R^3=H) showed one resonance which resonated at lower field than the one observed for the other deblocked isomer of 7b. Further, it was established that the diastereoisomer of d[Tp(s)A] with the higher δ_p-value was susceptible to digestion by snake venom phosphodiesterase. The ^{31}P-NMR[9] as well as the enzymatic digestion[10] data indicate that the diastereoisomer of d[Tp(s)A] derived from the higher R_f-diastereoisomer of 7b has the R_p-configuration and the other the S_p-configuration.

Table 1. Yields and other relevant data of dimers 7b (R^3=H).

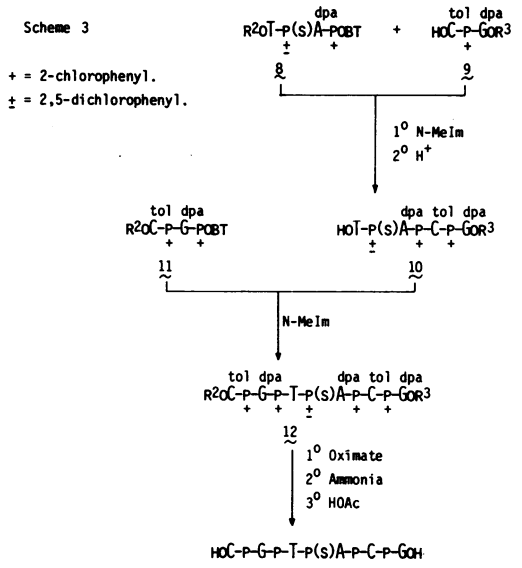
Dimers 7b (R^3=H)		Yield[a] (%)	δ_p-values[b] (ppm)	R_f values[c]
B^1	B^2			
A^{dpa}	A^{dpa}	66	62.3; 62.1	0.16; 0.11
A^{dpa}	G^{dpa}	67	62.0; 61.8	0.18; 0.12
A^{dpa}	T	70	62.3; 61.9	0.28; 0.24
C^{tol}	C^{tol}	67	62.6; 62.2	0.26; 0.18
G^{dpa}	A^{dpa}	60	62.4	0.40; 0.32
G^{dpa}	T	68	62.9; 62.8	0.31; 0.23
T	C^{tol}	65	62.5; 62.0	0.26; 0.20
T	T	65	62.5; 61.6	0.35; 0.28

a) based on 4. b) external reference 85% H_3PO_4; solvent $CDCl_3$. c) ethylacetate/methanol, 95/5, v/v.

Apart from the TA dimer, we also synthesized (according to Scheme 2) several other dimers of 7b (see Table 1). It can be seen that these dimers can be isolated in reasonable yields and, further, that the separation of the individual diastereoisomers of 7b can, in principle, easily be achieved by well-established column chromatography techniques.

The above data show that agent 1 can be converted with 1-hydroxy-6-nitrobenzotriazole 2b into the very effective phosphorylating agent 3b, which proved to be suitable for the regioselective introduction of a 3'-5'-phosphorothioate linkage between the *in situ* prepared intermediate 5b and the d-nucleoside 6 (R^3=H), to afford dimers 7b having a free 3'-OH group.

The applicability of dimers 7b will be demonstrated (see Scheme 3) by the synthesis in solution of the R_p and S_p-diastereoisomers of the hexamer d(CGTsACG). The higher R_f-diastereoisomer of 7b (B^1=T; B^2=A^{dpa}; R^2=DMTr; R^3=H) was phosphorylated with 2-chlorophenyl-O,O-bis[1-benzotriazolyl]phosphate[14] to afford the activated 3'-phosphotriester intermediate 8. The latter was coupled, in the presence of N-methylimidazole (N-MeIm), with the easily accessible[14] dimer 9 (R^3=benzoyl) to give, after work-up and subsequent removal of the DMTr-group (R^2) with benzenesulfonic acid[15], tetramer 10 in an overall yield (based on dimer 9) of 75%. Tetramer 10 was now condensed, in the presence of N-MeIm, with the similarly *in situ* prepared 3'-phosphorylated dimer 11 to give, after work-up and purification, the fully-protected hexamer 12 in a yield of 86% (based on 10). Hexamer 12 thus obtained was deblocked by a

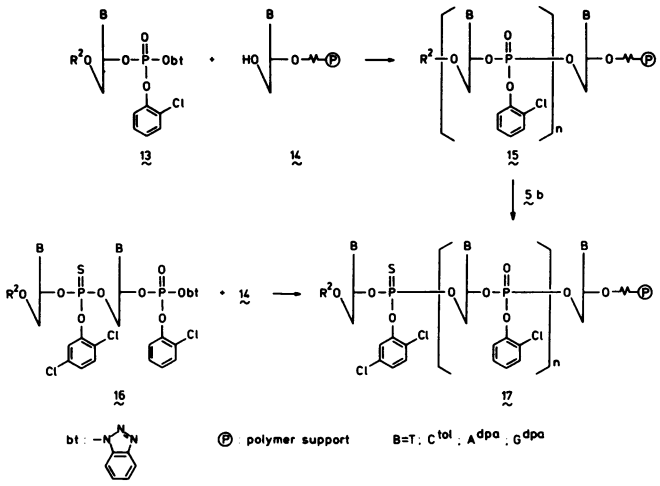


three step procedure (see Scheme 3) to give, after purification by gel-filtration column chromatography, the chirally pure R_p-isomer of d(CGTsACG). In the same way, starting from the lower R_f-diastereoisomer of 7b (B^1=T; B^2=A^{dpa}), the corresponding S_p-diastereoisomer of the same hexamer was obtained. The identity and homogeneity of the two hexamers was ascertained by ^{1}H- and ^{31}P-NMR spectroscopy as well as HPLC-analysis. Further, the R_p-isomer of the hexamer was, in contrast with the S_p-isomer, completely digested by venom phosphodiesterase. Finally, both isomers were completely desulfurized, as followed by ^{31}P-NMR spectroscopy, by iodine in pyridine[5] to afford the all-phosphate containing hexamer.

In conclusion, the data presented above show that dimers 7b are valuable synthons for the preparation of diastereoisomerically pure phosphorothioate-containing DNA fragments in solution.

Synthesis of DNA fragments containing phosphorothioate linkages on a solid support.

In recent papers[16], we showed that the activated 3'-phosphotriester derivatives 13 (R^2=DMTr; see Scheme 4), which are prepared *in situ* by phosphorylation of 4 (see Scheme 2) with 2-chlorophenyl-O,O-bis[1-benzotriazolyl]phosphate, could be coupled with 14 to give the immobilized dimer 15 (n=1). The coupling time of 13 with 14, in which the first d-nucleoside is anchored at the



Scheme 4

3'-position to controlled pore glass solid support (CPG/LCAA) by a succinate linkage, was 5 min using a fully automatic synthesizer. We now found that the coupling of the 3'-phosphorothioate intermediate 5b (Scheme 2) with the immobilized monomer 15 (n=0; R^2=H), to give 17 (n=0), could be realized in 10 min. The effectiveness of this step, as judged by UV-measurement of the released trityl-cation from 17 (R^2=DMTr; n=0), was more than 90%. The general applicability of using either monomers 5b or 13 in a solid-phase approach, will be illustrated by the synthesis of a mixture of diastereoisomers of the hexadecamer d(TCTAGATATCTGCTsTC). The steps to be performed for one complete elongation cycle, using the monomers 5b or 13 as the incoming d-nucleotides, are recorded in Table 2. Following this coupling protocol the immobilized monomer 15 (n=0; B=C^{tol}; R^2=DMTr) was firstly elongated (for coupling conditions, see step 3a in Table 2) with 13 (B=T; R^2=DMTr) to afford 15 (n=1). The latter dimer was then elongated (for coupling conditions, see step 3b in Table 2) with 5b (B^1=T) to afford 17 (n=1; R^2=DMTr), which was then elongated thirteen times with the appropriate monomer 13. The fully-protected and immobilized hexadecamer was completely deblocked under the conditions as previously indicated (see deprotection of 12 in Scheme 3). HPLC-analysis of the crude hexadecamer (see Figure 1) showed the presence of mainly one product, which was purified by Sephadex G-75 gel-filtration chromatography.

The same hexadecamer was also prepared by coupling (for coupling conditions, see step 3c in Table 2) of dimer 16 (B=T), which was obtained by phos-

Table 2. Steps to be performed for one complete chain elongation cycle on the solid support CPG/LCAA using a fully automatic DNA synthesizer[a].

Step	Manipulation	Solvents and reagents	Time (min)
1	detritylation	3% TCA in $ClCH_2CH_2Cl$	2
2	wash	acetonitrile	5
		a: 13/DIPEA/N-MeIm	5
3	coupling	b: 5b/DIPEA/N-MeIm	10
		c: 16/DIPEA/N-MeIm	5
4	wash	acetonitrile	2
5	capping	Ac_2O/Et_3N/N-MeIm/acetonitrile[b]	5
6	wash	acetonitrile	3
7	wash	$ClCH_2CH_2Cl$	3

a) Biosearch Inc., San Rafael, CA, USA. b) ratio: 4.5:4.5:1:30, v/v. TCA = trichloroacetic acid. DIPEA = N,N-diisopropylethylamine. N-MeIm = N-methylimidazole.

phorylation of the diastereoisomeric mixture of 7b (B^1=B^2=T; R^3=H) as mentioned before, with the immobilized d-nucleoside 14 (B=C^{tol}) to give 17 (n=1: sequence TTC). The latter immobilized trimer was elongated (for coupling conditions, see step 3a in Table 2) thirteen times with the monomer 13. Deprotection and purification of the immobilized product afforded the required hexadecamer having the same retention time as the previously prepared hexadecamer. Furthermore, the two differently prepared hexadecamers had the same retention times as the all-phosphate containing hexadecamer.

The above described methodology is to our knowledge the first successful

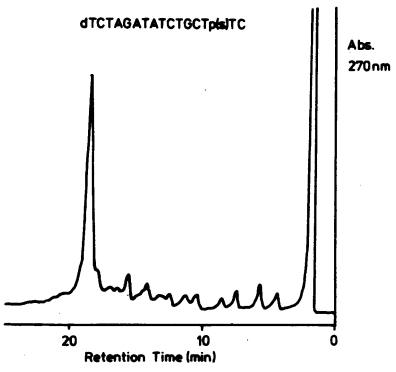


Figure 1. HPLC-analysis of the crude hexadecamer obtained by elongation of 14 with monomers 13 (14x) and 5b (1x).

solid-phase synthesis by a phosphotriester approach of DNA fragments having a phosphorothioate linkage.

Up to now[17], the solid-phase synthesis of similarly phosphate modified DNA fragments has been realized by two methods following a phosphite-triester approach. In the first method properly-protected d-nucleoside 3'-O-phosphite triesters are used for the elongation process. The introduction of a specific phosphorothioate linkage in the molecule is accomplished by thioylation, instead of oxidation, of the intermediate phosphite internucleotidic bond with sulfur. The thioylation step is, however, rather slow. For instance, a reaction time of 2 h using a suspension of sulfur in pyridine has been reported[17]. Similar results were obtained by performing the reaction with a homogeneous solution of sulfur in CS_2-pyridine. Recently[18], it has also been claimed that the rate of thioylation could be increased by performing the reaction at elevated temperature and by using a homogeneous solution of sulfur in 2,6-lutidine.

In the second method a presynthesized and properly-protected dimer having an intermediate phosphorothioate triester linkage, and carrying an activated 3'-phosphite function [i.e., 3'-O-(morpholinomethoxyphosphine)], has been applied[17] to realize the same goal. This method is, however, in several aspects less efficient than the one starting from dimer 16 in Scheme 2. First of all, the synthesis of the above described activated phosphite dimer is rather laborious and time-consuming. Furthermore, the formation of an intermediate 3'-5'-phosphite linkage between the above dimer and the 5'-OH of an immobilized d-nucleoside (e.g. 14 in Scheme 2) is rather slow (i.e., 45 min at 20°C). In our approach, the activated and *in situ* prepared dimers 16 can be coupled within a relatively short time (see step 3c in Table 2) with 14.

In conclusion, the data presented in this paper clearly show that the phosphorothioylating agent 3b is very effective for the synthesis in solution and on a solid support of phosphorothioate-containing nucleic acids by a phosphotriester approach.

EXPERIMENTAL

General methods and materials

Pyridine, dioxane and acetonitrile were dried by refluxing with CaH_2 for 16 h and then distilled.

Pyridine was redistilled from p-toluenesulfonyl chloride (50 g/l) and stored over molecular sieves 4Å. Dioxane was redistilled from $LiAlH_4$ (5 g/l) and stored over molecular sieves 4Å. N-Methylimidazole was distilled under reduced

pressure and stored over molecular sieves 4Å. N,N-diisopropylethylamine was distilled from chlorosulfonic acid and then redistilled from KOH-pellets. 1-Hydroxybenzotriazole (purchased from Janssen) was dried *in vacuo* (P_2O_5) for 70 h at 50°C. 1-Hydroxy-6-nitrobenzotriazole was prepared according to the procedure described by Curtius and Mayer[12] and dried *in vacuo* (P_2O_5) for 70 h at 40°C. Schleicher and Schüll DC Fertigfolien F 1500 LS 254 were used for TLC-analysis in the solvent system (a) CH_2Cl_2-MeOH (85:15, v/v) or (b) EtOAc-MeOH (95:5, v/v). The phosphorylation and coupling reactions were quenced on TLC-sheets with a mixture of pyridine-water (1:1, v/v). Short column chromatography was performed on Kieselgel 60 (230-400 mesh ASTM) suspended in CH_2Cl_2. The 5'-O-dimethoxytrityl derivatives of thymidine, N^4-(2-methylbenzoyl)-2'-deoxycytidine, N^6-diphenylacetyl-2'-deoxyadenosine and N^2-diphenylacetyl-2'-deoxyguanosine were prepared as described previously[19].

^{31}P-NMR spectra were recorded at 80.7 MHz with a Jeol J.N.M.-SX 200 spectrometer equipped with a J.E.C.-980 B computer, operating in the Fourier transform mode; proton noise decoupling was used. Chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard. ^{1}H-NMR spectra were recorded on a Bruker WM-300 spectrometer interfaced with an ASPECT-2000 computer and a real time pulser board. Chemical shifts are given relative to tetramethylammonium chloride.

High performance liquid chromatography was carried out on a Micromeritics liquid chromatograph 7000 B. U.V.-absorption (254 nm) was monitored with a Spectra Physics SP 8200 detector. Anion-exchange HPLC was performed on a Whatman Partisil-10SAX column (250 x 4.6 mm). The column was eluted with a linear gradient starting with buffer A (0.003 M KH_2PO_4, 20% CH_3CN U.V.-grade, pH 6.8) and applying 3% buffer B (0.65 M KH_2PO_4, 20% CH_3CN U.V.-grade, pH 6.8) per min. A flow of 1 ml/min at 50°C was standard. Solid phase DNA synthesis was performed on the Biosearch SAM-automatic DNA synthesizer.

Synthesis of 2,5-dichlorophenyl-O,O-bis 6-nitro-1-benzotriazolyl phosphorothioate 3b.

To a stirred suspension of 1-hydroxy-6-nitrobenzotriazole 2b (2.74 g; 15.2 mmole) in anhydrous dioxane (50 ml) was added pyridine (2.0 ml) followed by the dropwise addition of a solution of 2,5-dichlorophenyl phosphorodichloridothioate (2.22 g; 7.5 mmol; δ ^{31}P 54.8 ppm in CD_3CN) in anhydrous dioxane (15 ml). The solution was stirred for 4 h at 20°C and the pyridine-HCl salt was filtered off. The thus obtained 0.11 M stock solution of 3b (δ ^{31}P 65.4 ppm; CD_3CN) could be stored over several weeks at -20°C.

General procedure for synthesis of dinucleoside phosphorothioates 7b.

Step 1: preparation of intermediate 5b.
Compound 4 (1.0 mmole; B=T, C^{tol}, A^{dpa} or G^{dpa}) was dissolved in anhydrous pyridine and evaporated to dryness. To the dried residue was added 10 ml of a 0.11 M solution of the phosphorylating agent 3b (1.1 mmole) in dioxane and the mixture was stirred for 15 min at room temperature. TLC-analysis of the mixture showed that starting compound 4 was completely converted into intermediate 5b (R_f 0). The thus obtained solution of 5b (δ ^{31}P 63.3 ppm; CD_3CN) was immediately used for the synthesis of dimers 7b.

Step 2: preparation of dimers 7b.
The nucleoside 6 (R^3=H; B=T, C^{tol}, A^{dpa} or G^{dpa}; 1.25 mmole) was dissolved in anhydrous pyridine (15 ml) and concentrated to one third of the initial volume. To this solution was added 5b (1.0 mmole, see above) and N-methylimidazole (5.0 mmole). After stirring for 1 h at 20°C, TLC-analysis of the mixture showed the absence of 5b. The mixture was diluted with CH_2Cl_2 (100 ml), washed with 1 M TEAB (triethylammonium bicarbonate, 30 ml) and with water (3 x 30 ml). The CH_2Cl_2 layer was dried with $MgSO_4$ and concentrated under reduced pressure to an oil. The crude product was triturated with petroleum-ether (40-60°). The precipitate was removed by filtration, redissolved in CH_2Cl_2 and the minor impurities present in the crude product were removed by short column Kieselgel chromatography (12 g). The column was eluted with CH_2Cl_2 (applying a 0→10% gradient of MeOH). The fractions containing the two diastereoisomers of 7b were concentrated and precipitated from petroleum-ether (40-60°). The precipitate was filtered off and stored *in vacuo* (KOH-pellets). The yields, ^{31}P-chemical shifts and R_f-values of 8 dimers prepared as described above are recorded in Table 1. Note that a good separation of the two diastereoisomers of dimer 7b was obtained if solvent system (b), EtOAc-MeOH (95:5, v/v), was used.

Separation of the two diastereoisomers of dimer 7b (B^1=T; B^2=A^{dpa}) and assignment of their absolute configuration.

Dimer 7b (B^1=T; B^2=A^{dpa}; 0.64 mmole) obtained as a mixture of diastereoisomers (higher-R_f isomer: lower-R_f isomer = 3:1) was applied to a column (30 x 2 cm) of Kieselgel 60 (Lichroprep Si60, 40-63 μm, Merck) equilibrated in EtOAc. The column was eluted with EtOAc applying a MeOH gradient (0→10%, in 2 h) under a pressure of 1 atm (a flow of 5 ml per min). Fractions of 10 ml were collected. The fractions containing the optically pure higher-R_f isomer of 7b (B^1=T; B^2=A^{dpa}) were combined and concentrated to a small volume. The material

was precipitated from petroleum-ether (40-60°). The precipitate was filtered off and stored *in vacuo* (KOH-pellets). The same procedure was followed for the fractions containing the optically pure lower-R_f isomer.

Higher-R_f isomer: R_f 0.39 (solvent system b); δ ^{31}P ($CDCl_3$) 62.3 ppm; yield: 0.46 mmole.

Lower-R_f isomer : R_f 0.30 (solvent system b); δ ^{31}P ($CDCl_3$) 62.0 ppm; yield: 0.13 mmole.

The optically pure isomers of dimer 7b (100 mg; B^1=T; B^2=A^{dpa}) were separately treated with 5 ml of Ac_2O/2,6-lutidine/N-methylimidazole/CH_3CN (3:3:1:25, v/v) for 15 min. The solution was diluted with CH_2Cl_2 (20 ml) and washed with 1 M TEAB (20 ml) and with water (20 ml). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residual oil was redissolved in CH_2Cl_2 (5 ml) and precipitated from petroleum-ether (40-60°). The precipitates were collected by filtration and dried by coevaporation with acetonitrile. The fully protected diastereoisomers of dimer d[Tp(s)A] were deblocked by first treating them with a solution of 2-pyridinealdoxime (1.0 mmole) and N^1,N^1,N^3,N^3-tetramethylguanidine (0.9 mmole) in CH_3CN (3.0 ml). After 8 h, the solution was concentrated under reduced pressure. Concentrated aqueous ammonia (25%, 5 ml) was added to the residue and the reaction vessel was sealed and kept at 50°C for 48 h. Finally, the solution was concentrated and the nucleotide material was treated with aqueous AcOH (80%, 5 ml). After stirring for 30 min at 20°C, the solution was concentrated, the residue was redissolved in water (1 ml) and applied to a DEAE-Sephadex A25 column (20 x 2.5 cm). The column was eluted with a linear gradient of 0.05 M→0.4 M TEAB in 16 h at a flow rate of 60 ml per h. Fractions of 6 ml were collected. The appropriate fractions (Frs. 60-70, UV 260 nm-absorption) were combined, concentrated and the residue was passed through a column (10 x 2 cm) of Dowex 50W [cation-exchange resin (100-200 mesh, Na^+-form)] by elution with water. The nucleotide material was collected and lyophilized. By following the above described procedure the high-R_f isomer of dimer 7b (B^1=T; B^2=A^{dpa}) afforded 33 mg of a fully deblocked and optically pure isomer of d[Tp(s)A]. This isomer was identified as the R_p-diastereoisomer due to digestion in the presence of snake venom phosphodiesterase (HPLC and TLC-analysis). In the same way the low-R_f isomer afforded 32 mg optically pure d[Tp(s)A]-Sp which was not hydrolysed by snake venom phosphodiesterase.

R_p-Isomer of d[Tp(s)A]: δ ^{31}P (D_2O) 56.1 ppm,
δ ^1H (D_2O) 2.8 (H_1', t); 3.2 (H_1', t); 4.2 (H_6, s); 5.0 (H_2, s); 5.3 (H_8, s).

S_p-Isomer of d[Tp(s)A]: δ ^{31}P (D_2O) 55.3 ppm,
δ ^{1}H (D_2O) 2.8 (H_1', q); 3.2 (H_1', t); 4.2 (H_6, s); 5.0 (H_2, s); 5.3 (H_8, s).

Synthesis of d(CGTsACG) starting from the individual diastereoisomerically pure dinucleoside phosphorothioate of 7b (B^1=T; B^2=A^{dpa}; R^3=H).

The two diastereoisomers of dimer 7b (B^1=T; B^2=A^{dpa}) separated by Kieselgel chromatography were used as dimer blocks in the synthesis of d(CGTsACG). Diastereoisomerically pure dinucleoside phosphorothioate 7b (0.5 mmol; B^1=T. B^2=A^{dpa}; higher-R_f or lower-R_f isomer) was dried by coevaporation with anhydrous pyridine. 2-Chlorophenyl-O,O-bis[1-benzotriazolyl]phosphate 3a (0.55 mmole) in anhydrous dioxane (2.75 ml) was added to the dried residue and the mixture was stirred for 15 min at 20°C. TLC-analysis (system a) of the mixture showed the reaction to be complete: compound 7b was completely converted into 3'-phosphorylated dimer 8 (R^2=DMT; R_f 0). The thus obtained solution of 8 was immediately added to the partially protected dimer 9 (0.4 mmole; R^3=benzoyl) which was previously dried by coevaporation with anhydrous pyridine. N-methylimidazole (2.5 mmole) was added, and the reaction was stirred for 1.5 h at 20°C. TLC-analysis of the mixture showed the reaction to be complete. Excess 3'-phosphorylated dimer 8 was hydrolyzed by adding pyridine-water (1:1, v/v, 1 ml) to the mixture. The mixture was then diluted with CH_2Cl_2 (100 ml) and washed with 1 M triethylammonium bicarbonate (TEAB, 30 ml) and water (30 ml). The organic layer was dried with $MgSO_4$ and concentrated to an oil. The crude product was redissolved in CH_2Cl_2 (5 ml) and precipitated by the addition of petroleum-ether (40-60°). The precipitates were collected by filtration, redissolved in CH_2Cl_2 (5 ml) and applied to a short Kieselgel column (8 g). The column was eluted with CH_2Cl_2-MeOH (98→90:2→10, v/v) and the appropriate fractions were concentrated to a foam. The completely protected tetramer was obtained in a yield of 90% based on the 5'-OH dimer 9. The 5'-dimethoxytrityl group was removed by treating the fully protected tetramer with a solution (25 ml) of benzenesulfonic acid (2% by weight) in CH_2Cl_2-MeOH (7:3, v/v) during 5 min at 20°C. The reaction was quenched by adding aqueous $NaHCO_3$ (10% by weight, 12 ml). The organic layer was separated and washed with water, dried with $MgSO_4$ and concentrated to an oil under reduced pressure. The crude product was purified by short column Kieselgel chromatography (8 g) using the conditions as described earlier. Pure tetramer 10 (R^3=benzoyl) was obtained in a yield of 75% (based on 9). Chain extension of the tetramer (0.3 mmole) to obtain hexamer 12 was accomplished by coupling with 3'-phosphorylated dimer 11 (R^2=DMT; 0.4 mmole) using the same experimental conditions as described for the synthe-

sis of the tetramer. The individual R_p- and S_p-isomer of hexamer 12 were obtained in a yield of 86% and 80% (based on 10), respectively. Each isomer was completely deblocked as follows. To a stirred solution of the fully protected hexamer 12 (0.1 mmole; R^2=DMT; R^3=benzoyl) and 2-pyridinealdoxime (5.0 mmole) in 10 ml anhydrous dioxane-acetonitrile (1:1, v/v) was added N^1,N^1,N^3,N^3-tetramethylguanidine (4.5 mmole). After 4 h at 20°C, the solvent was removed by evaporation and the residue was redissolved in aqueous ammonia (25%, 50 ml). The reaction vessel was sealed and kept at 50°C for 48 h. Concentration of the mixture under reduced pressure was followed by treating the residue with aqueous acetic acid (80% by volume, 30 ml). After stirring for 30 min at 20°C, the acetic acid was removed under reduced pressure. The residue was redissolved in water (30 ml), extracted with CH_2Cl_2 (30 ml) and with ether (2 x 30 ml). The aqueous layer was concentrated and coevaporated three times with water. The crude hexamer d(CGTsACG) thus obtained was redissolved in water (3 ml), analyzed by anion-exchange HPLC and applied directly to a Sephadex G-50 column (2m x $3cm^2$). Elution was performed with 0.05 M NH_4OH at a flow-rate of 14 ml per hour. Fractions of 3 ml were collected, those containing the required product (HPLC-analysis) were pooled and concentrated to a small volume. The purified oligonucleotide was brought onto a column (10 x 2 cm) of Dowex 50W cation-exchange resin (100-200 mesh, Na^+-form) and eluted with water. The resulting aqueous solution was lyophilized. The purity of the oligonucleotide was monitored by anion-exchange HPLC. Yields, ^{31}P- and ^1H-NMR data obtained for the R_p- and S_p-isomers of d(CGTsACG) are as follows:

R_p-Isomer : Isolated yield: 141 mg (Na^+-salt).
δ ^{31}P (D_2O) : 55.45; -0.30; -0.39; -0.57 and -0.66 ppm,
δ ^1H (D_2O) : 5.37 (A-H_8, s); 4.83 and 4.74 (2 x G-H_8, s); 4.55 (A-H_2, s); 4.48 (C-H_6, d); 4.15 (C-H_5 and T-H_6, s); 3.14, 2.96, 2.81, 2.62, 2.53 and 2.35 (6 x H_1', m); 2.75 (C-H_6, d); 2.25 (D-H_6, m); -1.61 (T-CH_3, s).

S_p-Isomer : Isolated yield: 86 mg (Na^+-salt).
δ ^{31}P (D_2O) : 54.67; -0.27; -0.42; -0.54 and -0.72 ppm,
δ ^1H (D_2O) : 5.20 (A-H_8, s); 4.82 and 4.74 (2 x G-H_8, s); 4.59 (A-H_2, s); 4.48 (C-H_5, d); 4.18 (T-H_6, s); 4.15 (C-H_5, d); 3.14, 2.96, 2.73, 2.61, 2.50 and 2.16 (6 x H_1', m); 2.75 (C-H_6, d); 2.28 (C-H_6, d); -1.62 (T-CH_3, s).

The absolute configuration of the internucleotidic phosphorothioate linkage was confirmed by treatment of the R_p- and S_p-isomers with snake venom phosphodiesterase followed by anion-exchange HPLC-analysis. The R_p-isomer of d(CGTs-

ACG), which was prepared starting from the higher-R_f diastereoisomer of dimer 7 (B^1=T; B^2=A^{dpa}), was almost completely digested after incubation for 30 h with the enzyme. The S_p-isomer of d(CGTsACG), which was prepared starting from the lower-R_f isomer of dimer 7b (B^1=T; B^2=A^{dpa}), was only partially hydrolyzed under the same conditions. HPLC-analysis showed the presence of the nucleoside 5'-phosphates (dGMP and dCMP) and one peak with a retention time longer than the nucleoside 5'-phosphates but shorter than the hexamer. The latter peak was tentatively assigned as tetramer d(CGTsA).

Solid phase synthesis of DNA fragments having one internucleotidic phosphorothioate linkage.

Solid phase DNA synthesis was performed on a fully automatic DNA synthesizer (Biosearch Inc., San Rafael, CA, USA). Controlled pore glass/long chain alkylamine (CPG/LCAA, pore diameter 500 Å) loaded with the first nucleoside (capacity 30 μmol/g) was used as solid support. 40 Mg of CPG/LCAA was packed in a stainless steel column which is part of the automatic DNA synthesizer. 3'-Phosphotriester intermediates 13 (B=T, C^{tol}, A^{dpa} or G^{dpa}; Scheme 4) used in the elongation process, were prepared by adding phosphorylating agent 3a (R^1= H, 10 ml of a 0.11 M solution in CH_3CN) to nucleosides 4 (R^2=DMT; 1.0 mmole), which were previously dried by coevaporation with pyridine. 3'-Phosphorylated dimer 16 (R^2=DMT; 1.0 mmole) was prepared similarly, by adding reagent 3a (R^1= H, Scheme 1; 1.1 mmole) in CH_3CN (10 ml) to dimer 7b (R^2=DMT; R^3=H; 1.0 mmole of mixed isomers). After stirring for 10 min at 20°C, TLC-analysis of the mixtures showed the reactions to be complete. The 3'-phosphorothioate intermediate 5b (R^1=NO_2; Scheme 2) was prepared as described earlier. The thus obtained solutions of intermediates 13, 5b and 16 were transferred, under anhydrous conditions, into the reservoirs of the automatic DNA-synthesizer. The bottles containing solvents and reagents were kept under a slight argon pressure when operating the machine. All manipulations necessary for one complete elongation cycle, conversion of 15 (n=0, R^2=DMT) into 15 (n=1, R^2=DMT) are summarized in Table 2. Each step was performed by passing a continuous stream (flow 2-2.5 ml per min) of the solvents or reagents over the solid support. In the coupling step, intermediate 13 (0.1 mmole) in CH_3CN (1 ml) was mixed with N-methylimidazole/N,N-diisopropylethylamine/CH_3CN (2:1:4, v/v, 1 ml). By using a pump, the coupling mixture was recycled for 5 min through the column which was packed with solid support material. The efficiency of each elongation cycle was monitored by collecting the bright orange solution released during the detritylation step and measuring the absorbance at 498 nm.

Oligonucleotides having one internucleotide phosphorothioate linkage at a spe-

cific position, were prepared in two different ways. In one approach, only monomers were used in the elongation process. Introduction of a phosphorothioate linkage was realized by coupling (see step 3b in Table 2) with the appropriate 3'-phosphorothioate intermediate 5b (R^1=NO$_2$; 1 ml of a 0.1 M solution in dioxane). In the second approach, 3'-phosphorylated dimer 16 (mixed isomers) was coupled to 14 using the same conditions as normally applied for monomers 13 (step 3c in Table 1). The latter elongation step afforded trimer 17 (R^2=DMT, n=1) as a mixture of diastereoisomers. Further chain elongation with monomers 13 affords a fully protected phosphorothioate oligomer. Deprotection and isolation of the phosphorothioate oligonucleotides (diastereomeric mixture) was as follows. The immobilized and fully-protected DNA fragments were treated with a solution of 2-pyridinealdoxime (1.5 mmole, 183 mg) and N^1,N^1,N^3,N^3-tetramethylguanidine (1.35 mmole, 143 µl) in anhydrous CH_3CN (5 ml). After 16 h, the support was filtered off and washed with anhydrous CH_3CN (2 x 5 ml). Subsequently, the support was incubated with 25% aqueous ammonia (5 ml) at 50°C for 48 h in a sealed vessel. The resin was removed by filtration and washed with water (10 ml). The combined filtrates were evaporated to dryness and the resulting residue was treated with 5 ml of acetic acid/water (4:1, v/v) for 30 min at 20°C. The solution was concentrated under reduced pressure and the residue was coevaporated three times with water. Analysis of the crude product was performed by HPLC on a Whatman Partisil-10SAX column using the conditions as described in general methods and materials. Fully-deprotected phosphorothioate oligonucleotides obtained as a mixture of diastereoisomers were purified by Sephadex G-75 gel-filtration chromatography (column; 2m x 3cm^2). Elution was performed with 0.05 M NH_4OH at a flow-rate of 14 ml per h. Fractions of 3.0 ml were collected and analyzed by anion-exchange HPLC (no separation of the diastereoisomers was observed). The appropriate fractions were pooled and lyophilized to give a mixture of diastereoisomers of the phosphorothioate-containing oligonucleotide.

Digestion of oligonucleotides with snake venom phosphodiesterase.

Approximately 0.5 mg of the phosphorothioate-containing oligonucleotide was dissolved in 0.5 ml of 25 mM Tris-HCl, 5 mM $MgCl_2$, pH 8. 10 µL of snake venom phosphodiesterase (Boehringer) was added to the solution. After incubation for 24 h at 37°C, an aliquot of the digest was analyzed by HPLC (anion-exchange) under the conditions as described before.

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