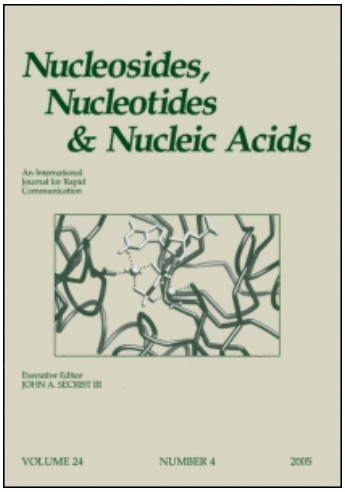

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SOLUTION PHASE SYNTHESIS OF DITHYMIDINE PHOSPHOROTHIOATE BY A PHOSPHOTRIESTER METHOD USING NEW *S*-PROTECTING GROUPS

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ABSTRACT. A phosphotriester method for the synthesis of dithymidine phosphorothioates with eight *S*-protecting groups has been investigated. Three of the *S*-protecting groups possesed catalytic activity, however side reactions occurred under deprotection. The best S-protecting group was 4-chloro-2-nitrobenzyl which could be removed with a minimum of side reactions (0.3 %). The coupling reagent PyFNOP (**11**) gave protected dithymidine phosphorothioate in 96 % yield after 15 min coupling.

Oligonucleoside phosphorothioates (POS-ODN) are among the most intensively investigated nuclease-resistant antisense analogues [1] and have been shown to have markedly antiviral properties and to inhibit a variety of oncogenes [2]. Recently a sequence of four consecutive guanosines (G-4 tract) within a larger POS-ODN was found to have antiproliferative effects and to prohibit HIV-infections *in vitro* [3]. Due to the many promising properties of POS-ODN there is an increasing need for scale up of POS-ODN synthesis from milligram to kilogram quantity. Presently, POS-ODN synthesis is being carried out on commercially available automated DNA synthesizers using either the phosphoramidite method [4-6] or the H-phosphonate method [7] using controlled pore glass as a solid support. During the last 4-5 years these methods have been under intense development for the manufacture of large quantities of POS-ODN as bulk pharmaceutical compounds for clinical evaluation. However it is still a matter of debate whether industrial production of large quantities of POS-ODN will operate by solid phase or solution phase chemistry [8-10].

The solid phase phosphoramidite method has been optimised to allow syntheses up to 5 mmol scale [11]. In solution the phosphotriester method is still the only method that is really

suitable for the large scale synthesis of oligonucleotides [12, 13] although the phosphoramidite approach has been used very recently for the large scale (30 mmol) synthesis of POS-dimers [14]. Furthermore the phosphotriester method has several advantages over other methods as 1) the nucleotide building blocks are very stable and easy to handle, 2) only a slight excess of nucleotide building blocks is necessary in the coupling step, 3) coupling reactions are not so sensible to water as water is removed by the excess of condensing agent, and 4) dimer, trimer and larger building blocks may be used and libraries of all 16 possible dimer blocks and all 64 possible trimer blocks can be synthesized on a large scale (i.e. block-synthesis) [12, 13].

Large scale synthesis of POS-ODN by the phosphotriester method has not yet been reported but very recently Barber *et al.* [15] and also Reese *et al.* [13] have reported on solution phase syntheses of POS-ODN by the phosphotriester method. Barber *et al.* synthesised a decathymidine phosphorothioate but observed side reactions during deprotection which led to breakage of the internucleotide bond (1.8 %) and formation of phosphate diester (0.9 %). Reese *et al.* synthesised dithymidine phosphorothioate in solution and did not observe any side reactions. However the method of Reese *et al.* seems less attractive for solution phase synthesis of POS-ODN, because the cyanoethyl *S*-protecting group is too labile for normal block synthesis. This prompted us to report our studies on a phosphotriester method for the synthesis of dithymidine phosphorothioates with new *S*-protecting groups.

Synthesis of nucleoside phosphorothioate monomers 6a-6h with new S-protecting groups

Froehler and Matteucci [16] introduced the ingenious idea of using catalytic phosphate protecting groups in nucleotide synthesis. Froehler and Matteucci used the 2-(*N*-methylimidazol-2-yl)phenyl as an efficient catalytic phosphate protecting group thereby taking advantage of the great rate acceleration achieved by anchimeric (neighboring group) assistance. Later Efimov *et al.* [17] used (2-pyridyl)methyl *N*-oxides as catalytic phosphate protecting groups in the synthesis of oligodeoxynucleotides by the phosphotriester method. Very recently the use of catalytic phosphate protecting groups has been applied to the synthesis of alkylphosphonates [18]. To the best of our knowledge catalytic protecting groups have not yet been used in the synthesis of POS-ODN. In this study we have investigated new *S*-protecting groups with catalytic activity.

The method we used to synthesize the nucleoside phosphorothioate monomers **6a-6h** with the new *S*-protecting groups is outlined in **figure 1** and is essential the same as used by Barber *et al.* [15]. The key step is alkylation of the *O*-cyanoethyl protected thymidine phosphorothioates **4** made by sulfur oxidation of the *H*-phosphonate **2**. The latter is easily accessible in almost quantitative yield either by a two step procedure from commercially available 5'-*O*-dimethoxytritylthymidine via the *H*-phosphonate **3** [19] or simply by addition of water and tetrazole to commercially available phosphoramidite **1**.

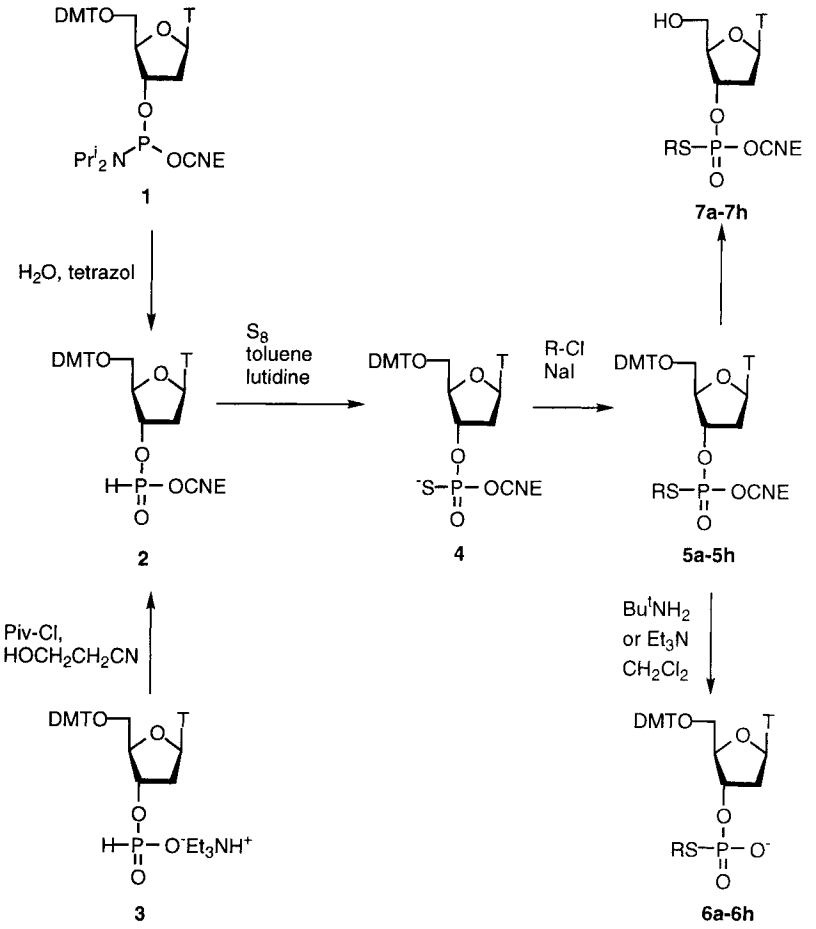
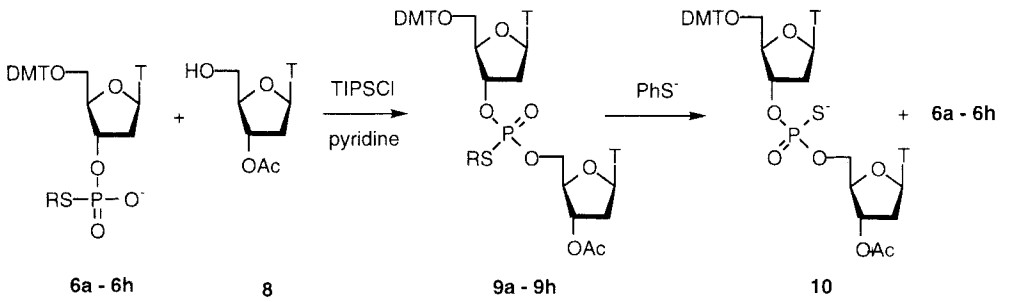


Figure 1. Synthesis of the phosphorothioate monomers **5a-5h** and **6a-6h**. R is shown in **fig. 2**.
4 was converted to the fully protected thymidine phosphorothioates **5a-5h** by alkylation, and the cyanoethyl group was easily and selectively removed by treatment with either 10% *tert*-butylamine in dry pyridine or 20% triethylamine in dry methylene chloride to give the ammonium salts **6a-6h** in almost quantitative yields.

Synthesis and deprotection of fully protected dithymidine phosphorothioates 9a-9h

The fully protected dithymidine phosphorothioates **9a-9h** were then synthesised in order to investigate whether the S-protecting groups 1) could be selectively removed to give **10** without side reactions and 2) gave any rate acceleration in the coupling step (i.e. could function as catalytic protecting groups). Our results are presented in the table in **Figure 2**.



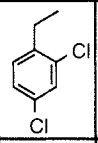
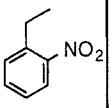
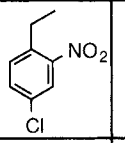
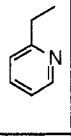
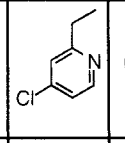
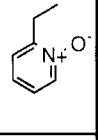
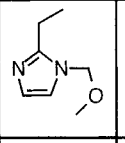
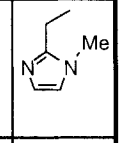
	a	b	c	d	e	f	g	h
R								
% 5'-cleavage	1.4	0.8	0.3	6.4	3.0	0.9	2.0	0.8
coupling time (min.)	8 > 60[a)]	8	8	< 2	8	< 2	< 4[a)]	< 2[a)]

Figure 2. Synthesis, *S*-deprotection and concomitant cleavage of the internucleoside linkage (5'-cleavage) in the phosphorothioate dimers **9a-9h**. The amount of 5'-cleavage was determined by ^{31}P-NMR. Couplings were performed as described in the experimental section. Deprotection was performed by treating the fully protected dimers **9a-9h** (0.1 mmol) with pyridine/triethylamine/thiophenol (0.1 ml : 0.1 ml : 0.1 ml) for 3-10 h. a) NMI was not added.

With the exception of the (2-pyridyl)methyl *N*-oxides dimer **9f**, the only side products detected by ^{31}P-NMR were the *S*-protected thymidine thiophosphate monomers **6a-6h** formed by cleavage of the internucleotide bridge by an attack of thiophenolate ions at the 5'-position of the dimer **9** (5'-cleavage in the table in **Figure 2**) [13]. The 2,4-dichlorobenzyl group in **9a** could not be removed selectively but gave rise to 1.4 % cleavage of the internucleotide bond which confirms the results found by Barber *et al.* (1.8 %) [15]. This is also in agreement with our recent results for phosphorodithioates [20]. Although the 2-nitrobenzyl group was presumed to be less labile than the 4-nitrobenzyl group [13], we found that the 2-nitrobenzyl group in **9b** gave rise to only 0.8 % 5'-cleavage, whereas the 4-nitrobenzyl group gave 1.1 % [13] or 0.8 % [15]. The 2,4-dinitrobenzyl group was reported unsuitable for synthesis of dithymidine phosphorothioate as the corresponding monomer **6** could not be obtained pure by the method in **figure 1** [15] and furthermore the use of such a monomer did

not lead to a detectable amount of the corresponding dimer **9** [13]. We reasoned, that the 4-chloro-2-nitrobenzyl group should have a lability between the 2-nitrobenzyl and 2,4-dinitrobenzyl groups. The monomer **6c** could be made in high yield and without the side reactions observed for the dinitrobenzyl monomer. The dimer **9c** could be deprotected with only 0.3 % 5'-cleavage which is very satisfactory. Among the potential catalytic protecting groups in **9d-9h**, the (*N*-methylimidazol-2-yl)methyl group in **9h** and the (2-pyridyl)methyl *N*-oxide group in **9f**, gave the lowest amount of 5'-cleavage, but the values (0.8 - 0.9%) were higher than that obtained for **9c**.

Couplings of **6a-6h** with 3'-acetyl protected thymidine **8** were performed in pyridine with the addition of 2,4,6-triisopropylbenzenesulfonyl chloride (TIPSCl) as coupling reagent and, apart from **6g** and **6h**, *N*-methylimidazole, and the coupling times are given in the table in **figure 2**. A modest rate enhancement was achieved when the 2-picolyl group was used (**6d**) relative to the use of 2,4-dichlorobenzyl group (**6a**). Unfortunately the 2-picolyl group could not be removed without serious cleavage of the internucleotide bond (6.4%). In order to make the *S*-protecting group more labile, the 4-chloro-2-picolyl group was introduced (**6e**). Although this protecting group showed to be more labile (only 3.0% cleavage of the internucleotide bond), we could not detect any rate enhancement when using **6e**. These results illustrate the delicate balance between making the *S*-protecting group more labile, which is done by placing electronegative substituents on the aromatic ring, and retaining the desired nucleophilic activity. Similar effects have been observed Efimov *et al.* [17]. We then tried the 2-picoline-*N*-oxide derivative **6f** and found that a moderate rate enhancement relative to the use of 2,4-dichlorobenzyl group **6a** was achieved. The 2-picolyl-*N*-oxide protecting group could be removed with only 0.9% cleavage of the internucleotide bond. Unfortunately the deprotection procedure also gave rise to 0.9% phosphate, which were also seen in an analogues study on phosphorodithioates [20]. There have previously been reported on side reactions when pyridine-*N*-oxides were used as nucleophilic catalysts in oligonucleotide synthesis [12]. The (*N*-methyl-2-imidazolyl)methyl group in **6h** gave the most significant rate enhancement relative to **6a**. Coupling with **6h** to dimer **9h** (without *N*-methylimidazole added in the coupling step) was complete in less than 2 min. whereas coupling with the "reference" **6a** to dimer **9a** took more than 60 min. under the same conditions. Furthermore as the (*N*-methyl-2-imidazolyl)methyl group in **9h** could be more selectively removed (0.8% 5'-cleavage) relative to the 2,4-dichlorobenzyl group in **9a** (1.4% 5'-cleavage), the monomer **6h** looked as a promising candidate for oligomer synthesis. However cleavage of the DMT protecting group in **5h** lead to very low isolated yields of the monomer **7h** and coupling between **7h** and **6h** gave no detectable amount of dimer.

The results described above led us to abandon the use of any of the investigated catalytic *S*-protecting groups for block-synthesis of POS-ODN in solution. Instead we turned our

attention to the more reactive coupling reagent 4-nitro-6-trifluoromethylbenzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyFNOP) **11** (**Figure 3**) [20-23] and found that the dimer **9c** could be synthesised fast (coupling reaction completed in less than 2 min.) and isolated in high yield (96 %).

The dimer **9c** was then completely deprotected by treatment with thiophenolate, 80 % aqueous acetic acid and finally conc. aqueous ammonia containing 0.01 M EDTA [24]. The ^{31}P-NMR spectrum of the crude product (**Figure 4**) exhibited, besides the major double peak at 55 ppm, assigned to the dithymidine phosphorothioate, only a minor peak (0.3%) at 16 ppm, assigned to DMT-deprotected **6c**. We could not detect any phosphate in the crude product in contrast to Barber *et. al.*[15]. We assign this to the fact that we used EDTA during the treatment with conc. ammonia [24].

In conclusion we have studied a phosphotriester method for the synthesis of dithymidine phosphorothioates using new *S*-protecting groups. For solution phase synthesis of dithymidine phosphorothioate the best results were obtained by using **6c** in combination with PyFNOP.

EXPERIMENTAL

5'-*O*-DMT-thymidin-3'-yl β-cyanoethyl N,N'-diisopropylphosphoramidite (**1**) was from Cruachem, 2,4-dichlorobenzyl chloride, 4-chloro-2-nitrobenzyl chloride, and 2-chloromethylpyridine hydrochloride were from Aldrich. 4-Chloro-2-chloromethylpyridine [25], 2-chloromethylpyridine-*N*-oxide [26, 27], 2-chloromethyl-*N*-methylimidazole hydrochloride [12], PyFNOP [22, 23], 1-hydroxy-4-nitro-6-trifluoromethylbenzotriazole [23], 3'-*O*-acetylthymidine **8** [28] were prepared as published. Acetonitrile (LAB-SCAN), dichloromethane (LAB-SCAN), pyridine (LAB-SCAN) and *N*-methylimidazole (Aldrich) were dried over 4Å molecular sieves (GRACE type 512). TLC was performed on silica 60 (Merck 5554 aluminium sheet), column chromatography on silica 60 (Merck 9385), packed in dichloromethane/ methanol/pyridine (98:1:1; v/v/v) and washed with 300-500 ml eluent. ^{31}P-NMR spectra were obtained on a JEOL FX 90 Q spectrometer at 36.24 MHz for ^{31}P in 5 mm tubes; chemical shifts are positive in the low-field direction, with external 85 % phosphoric acid as reference. ^{1}H spectra were recorded at 400 MHz, on a Varian XL-400 spectrometer, with internal reference TMS. ^{1}H and ^{31}P-NMR spectra were obtained in $CDCl_3$ if not otherwise stated. FAB MS spectra were obtained on a JEOL JMS-HX 110/HX 110A using nitrobenzylalkohol as matrix.

Triethylammonium *O*-[5'-*O*-(4,4'-dimethoxytrityl)thymidin-3'-yl] *O*-(β-cyanoethyl) phosphorothioate (4).

5'-*O*-DMT-thymidin-3'-yl β-cyanoethyl N,N'-diisopropylphosphoramidite **1** (4 g, 5.37 mmol) was dissolved in dry, degassed acetonitrile (19 ml) under nitrogen and water (0.3 ml,

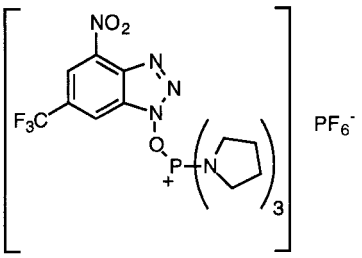


Figure 3. PyFNOP **11**

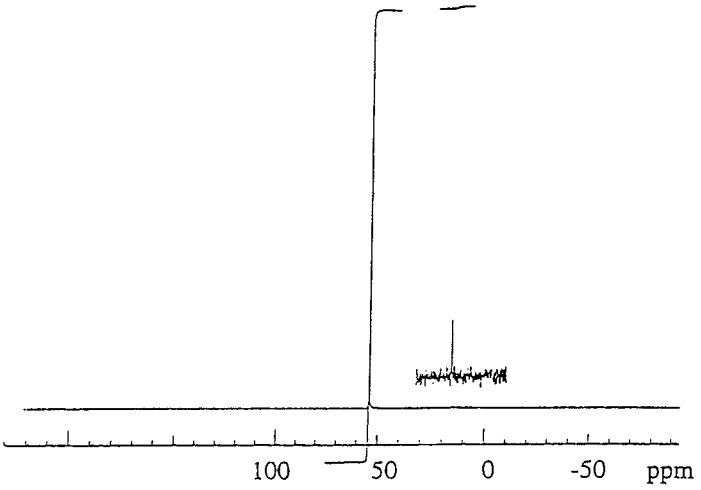


Figure 4. ^{31}P-NMR (D_2O) of crude, fully deprotected dithymidine phosphorothioate.

16.7 mmol) and tetrazole (0.75 g, 10 mmol) were added. After 15 min 0.5 M sulfur in benzene/2,6-lutidine (19:1) (25 ml) was added and the reaction mixture stirred at rt under nitrogen. After 60 min the reaction mixture was evaporated *in vacuo*, redissolved in ethyl acetate, filtrated and evaporated *in vacuo* to a white foam, which was purified by silica gel column chromatography using as eluent dichloromethane/ethyl acetate/ methanol/triethylamine (49:38:12:1; v/v/v/v). Fractions containing the product were combined and evaporated *in vacuo*.

Yield: 88 %. R_f = 0.26 (dichloromethane/ethyl acetate/ methanol/triethylamine, 49:38:12:1).

MS (FAB$^-$), 692[M-triethylammonium]$^-$.

^{31}P-NMR, δ 56.88 ppm (diastereoisomers not resolved).

General procedure for the preparation of *O*-[5'-*O*-(4,4'-dimethoxytrityl)thymidin-3'-yl] *O*-(β-cyanoethyl) *S*-(alkyl) phosphorothioates (5a-5h) (according to Barber *et al.* [26]).
To a stirred solution of the phosphorothioate diester (**4**) in dry acetonitrile (10 ml/mmol) was added 2,6-lutidine (5 mol eq.), NaI (3-6 mol eq.) and alkylation reagent (1.5-10 mol eq.). The reaction mixture was stirred at room temperature and the progress of the reaction was monitored by ^{31}P-NMR and TLC. When the reaction was completed (10 min to 16 h), the reaction mixture was diluted with dichloromethane (50 ml/mmol), washed with saturated aqueous sodium hydrogen carbonate (30 ml/mmol) and water (2x50 ml/mmol), dried over Na_2SO_4, filtered and evaporated *in vacuo*. The oily residue was purified by silica gel column chromatography using a stepwise gradient of methanol (0%-7% vol) in dichloromethane. Fractions containing the product were combined and evaporated *in vacuo*. The residue was dissolved in a small amount of methylene chloride and precipitated from petroleum ether to give a white powder that was dried over P_2O_5.

O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl] O-(β-cyanoethyl) S-(2,4-dichlorobenzyl) phosphorothioate (5a).
The reaction was carried out in the presence of 10 eq. 2,4-dichlorobenzyl chloride.
Yield: 66%. R_f = 0.71 (CH_2Cl_2/MeOH, 9:1). MS (FAB^+), 852$[M+H]^+$.
^{31}P-NMR, δ 27.19 ppm (diastereoisomers not resolved).
^{1}H-NMR, δ 8.24 (bs, 1H, NH), 7.55 (2d, 1H, H_6), 7.40 and 6.80 (2m, 16H, arom, DMT and dichlorobenzyl), 6.42 (m, 1H, $H_{1'}$), 5.22 (m, 1H, $H_{3'}$), 4.27-4.05 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.80 (s, 6H, OCH_3), 3.47 and 3.32 (2m, 2H, $H_{5'}$), 2.72-2.31 (m, 4H, CH_2CN and $H_{2'}$), 1.40 [2d, 3H, CH_3 (thymine)].

O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl] O-(β-cyanoethyl) S-(2-nitrobenzyl) phosphorothioate (5b).
The reaction was carried out in the presence of 10 eq. 2-nitrobenzyl chloride.
Yield: 45%. R_f = 0.73 (CH_2Cl_2/MeOH, 9:1).
^{31}P-NMR, δ 27.31 ppm (diastereoisomers not resolved).
^{1}H-NMR, δ 9.95 (bs, 1H, NH), 8.1 (2d, 1H, H_6), 7.6 and 6.80 (2m, 16H, arom, DMT and dichlorobenzyl), 6.35 (m, 1H, $H_{1'}$), 5.2 (m, 1H, $H_{3'}$), 4.45-4.05 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.80 (s, 6H, OCH_3), 3.47 and 3.32 (2m, 2H, $H_{5'}$), 2.7-2.3 (m, 4H, CH_2CN and $H_{2'}$), 1.4 [2d, 3H, CH_3 (thymine)].

O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl] O-(β-cyanoethyl) S-(4-chloro-2-nitrobenzyl) phosphorothioate (5c).
The reaction was carried out in the presence of 1.5 eq. 4-chloro-2-nitrobenzyl chloride.
Yield: 90% (from **1**). R_f = 0.70 (CH_2Cl_2/MeOH, 9:1). MS (FAB^+), 865$[M+H]^+$.

^{31}P-NMR, δ 27.02 ppm (diastereoisomers not resolved).
^{1}H-NMR, δ 9.1 and 8.8 (2bs, 1H, NH), 8.0 (2d, 1H, H_6), 7.50 and 6.80 (2m, 16H, arom, DMT and dichlorobenzyl), 6.3 (m, 1H, $H_{1'}$), 5.1 (m, 1H, $H_{3'}$), 4.35-4.0 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.80 (s, 6H, OCH_3), 3.4 and 3.3 (2m, 2H, $H_{5'}$), 2.6-2.3 (m, 4H, CH_2CN and $H_{2'}$), 1.40 [2d, 3H, CH_3 (thymine)].

***O*-[5'-*O*-(4,4'-Dimethoxytrityl)thymidin-3'-yl] *O*-(β-cyanoethyl) *S*-((2-pyridyl)methyl phosphorothioate (5d).**

The reaction was carried out in the presence of 4.3 eq. 2-chloromethylpyridine hydrochloride.
Yield: 43%. R_f = 0.61 (CH_2Cl_2/MeOH, 9:1). MS (FAB$^+$), 784 $[M+H]^+$.
^{31}P-NMR, δ 27.59 and 27.47 ppm (diastereoisomers).
^{1}H-NMR, δ 8.90 (2s, 1H, NH), 8.70, 7.65 and 7.35 (3m, 4H arom, picolyl), 7.46 (2s, 1H, H_6), 7.25 and 6.80 (2m, 13H, arom, DMT), 6.40 (m, 1H, $H_{1'}$), 5.25 (m, 1H, $H_{3'}$), 4.30-4.10 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.78 (s, 6H, OCH_3), 3.47 and 3.32 (2m, 2H, $H_{5'}$), 2.70-2.40 (m, 4H, CH_2CN and $H_{2'}$), 1.45 [2d, 3H, CH_3 (thymine)].

***O*-[5'-*O*-(4,4'-Dimethoxytrityl)thymidin-3'-yl] *O*-(β-cyanoethyl) *S*-(4-chloro-2-pyridyl)-methyl phosphorothioate (5e).**

The reaction was carried out in the presence of 3 eq. 4-chloro-2-chloromethylpyridine.
Yield: 85%. R_f = 0.66 (CH_2Cl_2/MeOH, 9:1). MS (FAB$^+$), 818 $[M+H]^+$.
^{1}P-NMR, δ 27.23 ppm (diastereoisomers not resolved).
^{1}H-NMR, δ 8.70 (2s, 1H, NH), 8.70 and 7.35 (2m, 3H arom, picolyl), 7.46 (2s, 1H, H_6), 7.25 and 6.80 (2m, 13H, arom, DMT), 6.40 (m, 1H, $H_{1'}$), 5.25 (m, 1H, $H_{3'}$), 4.15-4.05 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.78 (s, 6H, OCH_3), 3.47 and 3.32 (2m, 2H, $H_{5'}$), 2.70-2.40 (m, 4H, CH_2CN and $H_{2'}$), 1.45 [2d, 3H, CH_3 (thymine)].

***O*-[5'-*O*-(4,4'-Dimethoxytrityl)thymidin-3'-yl] *O*-(β-cyanoethyl) *S*-(2-pyridyl)methyl *N*-oxide phosphorothioate (5f).**

The reaction was carried out in the presence of 2-chloromethylpyridine-*N*-oxide.
Yield: 70%. R_f = 0.50 (CH_2Cl_2/MeOH, 9:1). MS (FAB$^+$), 800 $[M+H]^+$.
^{31}P-NMR, δ 28.52 and 28.23 ppm (diastereoisomers).
^{1}H-NMR, 8.90 (2s, 1H, NH), 8.70, 7.65 and 7.35 (3m, 4H arom, picolyl), 7.46 (2s, 1H, H_6), 7.25 and 6.80 (2m, 13H, arom, DMT), 6.40 (m, 1H, $H_{1'}$), 5.25 (m, 1H, $H_{3'}$), 4.30-4.10 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.78 (s, 6H, OCH_3), 3.47 and 3.32 (2m, 2H, $H_{5'}$), 2.70-2.40 (m, 4H, CH_2CN and $H_{2'}$), 1.45 [2d, 3H, CH_3 (thymine)].

O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl] O-(β-cyanoethyl) S-(N-methoxymethylene-2-imidazolyl)methyl phosphorothioate (5g).

The reaction was carried out in the presence of 2 eq. 2-chloromethyl-*N*-methoxymethylimidazole hydrochloride.

Yield: 30%. Rf = 0.64 (CH_2Cl_2/MeOH, 9:1). MS (FAB^+), 842 $[M+Na]^+$.

^{31}P-NMR, δ 26.58 and 26.46 ppm (diastereoisomers).

^{1}H-NMR, δ 10.2 (bs, 1H, NH), 7.5 (s, 1H, H_6), 7.25 and 6.80 (2m, 15H, arom, DMT and imidazole), 6.40 (m, 1H, $H_{1'}$), 5.25 (m, 1H, $H_{3'}$), 5.2 (s, 2H, OCH_2N-imidazole), 4.30-4.10 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.78 (s, 6H, OCH_3), 3.65 [2s, 3H, OCH_3], 3.47 and 3.32 (2m, 2H, $H_{5'}$), 2.70-2.40 (m, 4H, CH_2CN and $H_{2'}$), 1.4 [2s, 3H, CH_3 (thymine)].

O-[5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl] O-(β-cyanoethyl) S-(N-methyl-2-imidazolyl)methyl phosphorothioate (5h).

The reaction was carried out in the presence of 2-chloromethyl-*N*-methylimidazole hydrochloride.

Yield: 62%. Rf = 0.50 (CH_2Cl_2/MeOH, 9:1). MS (FAB^+), 807 $[M+H]^+$.

^{31}P-NMR, δ 26.58 and 26.46 ppm (diastereoisomers).

^{1}H-NMR, δ 9.5 (bs, 1H, NH), 7.5 (bs, 1H, H_6), 7.25 and 6.80 (2m, 15H, arom, DMT and imidazole), 6.40 (m, 1H, $H_{1'}$), 5.25 (m, 1H, $H_{3'}$), 4.30-4.10 (m, 5H, P-OCH_2, P-SCH_2, and $H_{4'}$), 3.78 (s, 6H, OCH_3), 3.65 [s, 3H, CH_3 (*N*-methyl imidazole)], 3.47 and 3.32 (2m, 2H, $H_{5'}$), 2.70-2.40 (m, 4H, CH_2CN and $H_{2'}$), 1.45 [2d, 3H, CH_3 (thymine)].

General procedure for the removal of the cyanoethyl group in the preparation of *tert.*-butylammonium or triethylammonium O-[5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl] S-(alkyl) phosphorothioates (6a-6h).

The following compounds were prepared according to Barber et al. [15], or as described.

***tert.*-Butylammonium O-[5'-O-(4,4'-dimethoxytrityl)-thymidin-3'-yl] S-(2,4-dichlorobenzyl) phosphorothioate (6a).**

MS (FAB^-), 797 [M-*tert*-butylammonium]$^-$. ^{31}P-NMR, δ 14.2 ppm.

***tert.*-Butylammonium O-[5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl] S-(2-nitrobenzyl) phosphorothioate (6b).**

MS (FAB^-), 772.5 [M-*tert*-butylammonium]$^-$. ^{31}P-NMR, δ 16.3 ppm.

Triethylammonium O-[5'-O-(4,4'-dimethoxytrityl)thymidin-3'-yl] S-(4-chloro-2-nitrobenzyl) phosphorothioate (6c).

To a stirred solution of **5c** in anhydrous dichloromethane (5 ml/mmol) was added anhydrous

triethylamine (1.25 ml/mmol) and the reaction mixture was stirred at room temperature overnight and the reaction mixture evaporated *in vacuo*. The residue was dissolved in a small amount of dichloromethane and precipitated at 0°C from petroleum ether to give a white powder. After centrifugation the supernatant was discarded and the precipitate was suspended in petroleum ether and centrifuged again. The precipitate was dried *in vacuo* and was used without further purification.
MS (FAB$^-$), 809.8 [M-triethylammonium]$^-$. ^{31}P-NMR, δ 14.5 ppm.

***tert.*-Butylammonium *O*-[5'-*O*-(4,4'-dimethoxytrityl)thymidin-3'-yl] *S*-(2-pyridyl)-methyl phosphorothioate (6d).**
MS (FAB$^-$), 727.9 [M-*tert*-butylammonium]$^-$. ^{31}P-NMR, δ 14.7 ppm.

***tert.*-Butylammonium *O*-[5'-*O*-(4,4'-dimethoxytrityl)thymidin-3'-yl] *S*-(4-chloro-2-pyridyl)methyl phosphorothioate (6e).**
MS (FAB$^-$), 764 [M-*tert*-butylammonium]$^-$. ^{31}P-NMR, δ 15.96 ppm.

***tert.*-Butylammonium *O*-[5'-*O*-(4,4'-dimethoxytrityl)thymidin-3'-yl] *S*-(2-pyridylmethyl *N*-oxide) phosphorothioate (6f).**
MS (FAB$^-$), 746 [M-*tert*-butylammonium]$^-$. ^{31}P-NMR, δ 14.7 ppm.

***tert.*-Butylammonium *O*-[5'-*O*-(4,4'-dimethoxytrityl)thymidin-3'-yl] *S*-(*N*-methoxy-methylene-2-imidazolyl)methyl phosphorothioate (6g).**
MS (FAB$^-$), 761.4 [M-*tert*-butylammonium]$^-$. ^{31}P-NMR, δ 14.3 ppm.

***tert.*-Butylammonium *O*-[5'-*O*-(4,4'-dimethoxytrityl)thymidin-3'-yl] *S*-(*N*-methyl-2-imidazolylmethyl) phosphorothioate (6h).**
MS (FAB$^-$), 733 [M-*tert*-butylammonium]$^-$. ^{31}P-NMR, δ 14.2 ppm.

General procedure for the coupling to the dimers 9a-9h (according to Barber *et al.* [15]).
3'-Acetylthymidine and **6a-6h** (1.2 mol eq) were dried by evaporation twice from dry pyridine (10 ml/mmol) and redissolved in dry pyridine under nitrogen(10 ml/mmol of 3'-acetylthymidine). To the stirred reaction mixture were successively added *N*-methyl imidazole (10 mol eq; except for **6g** and **6h**) and 2,4,6-triisopropylbenzenesulfonyl chloride (3 mol eq). After the completion of the reaction (2 - 60 min as judged by ^{31}P-NMR and TLC) the reaction mixture was diluted with an equal volume of pyridine, quenched by addition of a solution of saturated aqueous sodium hydrogen carbonate (5 ml/mmol) and after stirring at room temperature for 30 min. the reaction mixture was dissolved in dichloromethane (100

ml/mmol) and washed with a solution of saturated aqueous sodium hydrogen carbonate (200 ml/mmol). The aqueous phase was extracted with dichloromethane (2 x 100 ml/mmol), and the combined organic phases was washed with water (200 ml/mmol), dried over Na_2SO_4, filtered and evaporated *in vacuo*. The residue was purified by silica gel column chromatography using a stepwise gradient of methanol (0%-7% v/v) in dichloromethane. Fractions containing the product were combined and evaporated *in vacuo*. The residue was dissolved in a small amount of dichloromethane and precipitated from petroleum ether to give a white powder that was dried *in vacuo*. The products were ca. 1:1 mixtures of two diastereomers which were not separated.

dimer 9a. Yield: 95 %. ^{31}P NMR: δ 27.63 og 27.39 ppm. R_f = 0.52 (CH_2Cl_2/MeOH, 9:1).
dimer 9b. Yield: 81 %. ^{31}P NMR: δ 27.67 og 27.47 ppm.
dimer 9c. Yield: 48 %. ^{31}P NMR: δ 27.43 ppm. R_f = 0.64 (CH_2Cl_2/MeOH, 9:1).
dimer 9d. Yield: 88 %. ^{31}P NMR: δ 28.03 og 27.80 ppm. R_f = 0.47 (CH_2Cl_2/MeOH, 9:1).
dimer 9e. Yield: 65 %. ^{31}P NMR: δ 27.71 og 27.59 ppm. R_f = 0.50 (CH_2Cl_2/MeOH, 9:1).
dimer 9f. ^{31}P NMR: δ 28.72 og 29.13 ppm.
dimer 9g. *N*-methylimidazole not added in the coupling step.
Yield: 78 %. ^{31}P NMR: δ 27.27 og 26.78 ppm. R_f = 0.43 (CH_2Cl_2/MeOH, 9:1).
dimer 9h. *N*-methylimidazole not added in the coupling step.
Yield: 49 %. ^{31}P NMR: δ 27.31 og 26.88 ppm.

Synthesis of the dimer 9c using PyFNOP.

Monomer **6c** (400 mg; 0.44 mmol) and 3'-acetylthymidine **8** (150 mg; 0.53 mmol) were dried by evaporation from dry acetonitrile (2x5 ml), redissolved in dry acetonitrile (2.2 ml) under nitrogen and dry *N*-methylimidazole (0.35 ml; 4.4 mmol) was added under nitrogen and then PyFNOP (557 mg; 0.88 mmol). The progress of the reaction was followed by TLC and ^{31}P NMR. After 15 min. the reaction was quenched by addition of saturated sodium hydrogen carbonate (0.25 ml) and the mixture taken up in ethyl acetate (100 ml) and washed with saturated sodium hydrogen carbonate (4 x 25 ml). The aqueous phase was back extracted with ethyl acetate (2 x 50 ml) and the combined organic phases washed with saturated sodium chloride (2 x 50 ml), dried over anhydrous sodium sulfate and evaporated *in vacuo*. The orange foam was purified by chromatography on silica using as eluent methanol/dichloromethane/ethyl acetate (5:63:32, v/v/v). Fractions containing the product were evaporated and dried *in vacuo*.
Yield: 96 %. ^{31}P NMR: δ 27.43 ppm.

Deprotection of dimer 9c.

Fully protected dimer **9c** (65 mg, 0.06 mmol) was treated with a mixture of thiophenol/triethylamine/pyridine (0.1 ml: 0.1 ml: 0.1 ml) for 1h at room temperature and the reaction mixture was evaporated *in vacuo*. The residue was trituated with diethyl ether (10 ml) and the white powder washed with diethyl ether (3 x 15 ml) and then dried by evaporation *in vacuo*. The white powder was dissolved in 80% acetic acid (2 ml), and the reaction mixture was stirred at room temperature for 20 minutes and then neutralized with conc. ammonia containing 0.01 M EDTA (1.8 ml). The aqueous layer was washed with diethyl ether (3 x 10 ml) and lyophilized. The residue was then treated with concentrated aqueous ammonia (2 ml) for 3 h at room temperature and lyophilized.

^{31}P NMR (D_2O): δ 55.46 and 55.22 ppm.

References.

1. Stein, C.A., Tonkinson, J.L. and Yakubov, L., *Pharmacol. Ther.*, **1991**, 52, 365-384.
2. Milligan, J.F., Matteucci, M. and Martin, J.C., *J. Med. Chem.*, **1993**, 36, 1923-1937.
3. Wyatt, J. R., Vickers, T. A., Roberson, J. L., Buckheit, R. W., Klimkait, T., Debaets, E., Davis, P. W., Rayner, B., Imbach, J.-L. and Ecker, D. J. *Proc. Natl. Acad. Sci. USA*, **1994**, 91, 1356-1360.
4. Stec, W.J., Zon, G., Egan, W. and Stec, B., *J. Am. Chem. Soc.* **1984**, 106, 6077-6079.
5. Iyer, R.P., Egan, W., Regan, J.B. and Beucage, S.L., *J. Am. Chem. Soc.*, **1990**, 112, 1253-1254.
6. Vu, H. and Hirschbein, B., *Tetrahedron Lett.*, **1991**, 31, 1953-1956.
7. Andrus, A. and Zon, G., *Nucleic Acids Res. Symposium Series*, **1988**, 20, 121-122.

8a. Zon, G., In: *Protocols for oligonucleotides and analogs.* S. Agrawal, ed. (Humana Press, Totowa, NJ), **1993**, 165-189.

8b. Zon, G. and Geiser, G.T., *Anticancer Drug Des.*, **1991**, 6, 539-568.

9. Seliger, H., In: *Protocols for oligonucleotides and analogs.* S. Agrawal, ed. (Humana Press, Totowa, NJ), **1993**, 391-435.
10. Sinha, N.D., In: *Protocols for oligonucleotides and analogs.* S. Agrawal, ed. (Humana Press, Totowa, NJ), **1993**, 437-463.
11. Padmapriya, A.A., Jinyan, T. and Agrawal, S. *Antisense Res. Dev.*, **1994**, 4, 185-199.
12. Reese, C. B. and Pei-Zhuo, Z., *J. Chem. Soc. Perkin Trans., I* ,**1993**, 2291-2301.
13. Liu, X. and Reese, C. B., *J. Chem. Soc. Perkin Trans. I*, **1995**, 112, 1685-1694.
14. Ravikumar, V. T., Krotz, A. H. and Cole, D. L., *Tetrahedron Lett.*, **1995**, 36, 6587-6590.
15. Barber, I., Imbach, J.-L. and Rayner, B., *Antisense Res. Dev.*, **1995**, 5, 39-47.
16. Froehler, B. C. and Matteucci, M. D., *J. Am. Chem. Soc.*, **1985**, 107, 278-279.

17. Efimov, V. A., Buryakova, A. A., Dubey, I. Y., Polushin, N. N., Chakhmakhcheva, O. G. and Ovchinnikov, Y. A., *Nucleic Acids Res.*, **1986**, 14, 6525-6540.
18. Szabó, T., Kers, A. and Stawinski, J., *Nucleic Acids Res.*, **1995**, 23, 893-900.
19. Jankowska, J., Sobkowski, M., Stawinski, J. and Kraszewski, A., *Tetrahedron Lett.*, **1994**, 35, 3355-3358.
20. Kehler, J., Püschl, A. and Dahl, O., *Nucleosides and Nucleotides*, submitted.
21. Kehler, J., Püschl, A. and Dahl, O., *Tetrahedron Lett.,* in press.
22. Kehler, J., Püschl, A. and Dahl, O., *Acta Chem. Scand.*, in press.
23. Wijkmans, J. C. H. M., Blok, F. A. A., van der Marel, G. A., van Boom, J. H. and Bloemhoff, W., *Tetrahedron Lett.*, **1995**, 36, 4643-4646.
24. Kodra, J. T., Kehler, J. and Dahl, O., *Nucleic Acids Res.*, **1995**, 23, 3349-3350.
25. Barnes, J. H.; Hartley, F. R.; Jones, C. E. L., *Tetrahedron*, **1982**, 38, 3277-3280.
26. Tilley, J. W.; Levitan, P.; Kierstad, R. W, *Heterocyclic Chem.*, **1979**, 16, 333-337.
27. Mizuno, Y.; Endo, T.; Takahashi, A.; Inaki, A., *Chem. Pharm. Bull.*, **1980**, 28, 3041.
28. Horwitz, J. P.; Urbanski, J. A.; Chua, J., *J. Org. Chem.*, **1962**, 27, 3300-3302.

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