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A New Strategy for the Solid-Phase Synthesis of 5'-Thiolated Oligodeoxynucleotides

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Abstract: A novel procedure is described for the introduction of a masked thiol function at the 5'-terminus of oligodeoxynucleotides. An acetyl-protected thiol linker phosphoramidite has been prepared which can be coupled efficiently to fully protected oligonucleotides using standard solid-phase techniques. Our strategy is elaborated for oligothymidylates and is further illustrated for oligodeoxynucleotides which are base-protected with the 2-(acetoxymethyl)benzoyl group. Rapid base-deprotection can be accomplished in methanolic potassium carbonate which also effects saponification of the thioacetyl ester. The resultant free thiol is reacted *in situ* with dithiodipyridine to afford pyridyldisulfide-derivatized oligodeoxynucleotides.

Introduction

In the past decade, enormous improvements have been achieved in oligonucleotide synthesis.[1] These have not only resulted in an easy accessibility of a wide variety of modified oligonucleotides but also have stimulated the development of several oligonucleotide conjugates for various applications.[2] Our interest is focused on conjugates of monoclonal antibodies and (modified) oligonucleotides, which are intended for application in a novel strategy in radioimmunotherapy of cancer, the so-called DNA-DNA pretargeting regimen.[3] In this approach radiolabeled complementary oligonucleotides are targeted to tumor sites after saturation of the tumor with antibody-DNA conjugates. For the preparation of the conjugates, thiol group containing oligonucleotides are required which can be conveniently linked with maleimide-derivatized monoclonal antibodies.

Several studies have been published on the introduction of thiol groups into synthetic oligodeoxynucleotides. Some of these reports deal with functionalization of 5'-amino containing oligonucleotides, with commercially available reagents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP)[4,5] or N-acetyl-homocysteine lactone.[6]

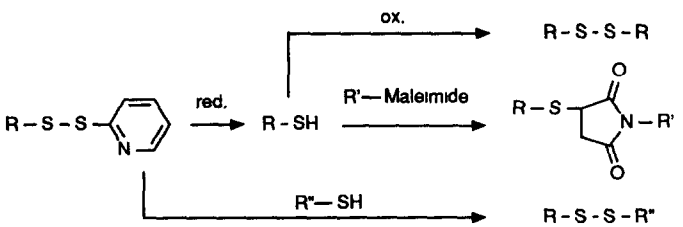
In more direct approaches, in which the thiol group is already incorporated during solid-phase DNA synthesis, two strategies can be distinguished. The first approach aims to produce oligonucleotides that contain a 3'-terminal thiol group.[7-11] To this end, modified solid supports have to be prepared containing a disulfide linkage. The second strategy involves the preparation of oligonucleotides containing a thiol group at their 5'-termini. The thiol functions are introduced as trityl-protected thiol building blocks which can be linked to oligonucleotides assembled using either phosphoramidite or H-phosphonate chemistry.[12,13]

In analogy with the latter approach, a trityl-protected thiol linker phosphoramidite was synthesized by us with the aim to introduce a masked thiol function at the end of the solid-phase DNA synthesis. However, in our hands the coupling of the trityl-thio amidite to the solid-support bound oligonucleotide was found to proceed with low efficiency under various reaction conditions.[14] Moreover, the removal of excess dithioerythritol (DTE), used to liberate the thiol group after deprotection of the trityl function with silver nitrate, turned out to be a rather tedious procedure.

The drawbacks of the trityl protective group prompted us to develop a new strategy for functionalizing oligonucleotides with a protected thiol group. In this paper, we wish to report on the use of an acetyl-protected thiol linker which can be smoothly introduced into oligonucleotides on solid support using standard phosphoramidite chemistry. Deprotection of the oligonucleotides is easily accomplished with methanolic potassium carbonate, while the presence of dithiodipyridine (DTDP) in the deprotection mixture ensures an efficient conversion of the liberated thiol into a pyridyldisulfide function. It is demonstrated that in this way DNA fragments are obtained which contain a thiopyridyl-masked thiol group.

Results and discussion

As a consequence of their susceptibility to oxidation, thiol containing oligonucleotides are usually prepared and stored in a protected form. Since disulfide formation can be easily reversed by reduction, it is often useful to obtain the thiols in their oxidized state, either as symmetrical disulfides or, more generally, as pyridyldisulfides (Scheme 1). Oligonucleotides derivatized with a thiopyridyl-masked thiol linker have been described before.[8-11] In all those cases, however, the pyridyldisulfide linkage has been generated upon treatment of the free thiol with dithiodipyridine (DTDP), only after deprotection of the oligonucleotide and HPLC purification.



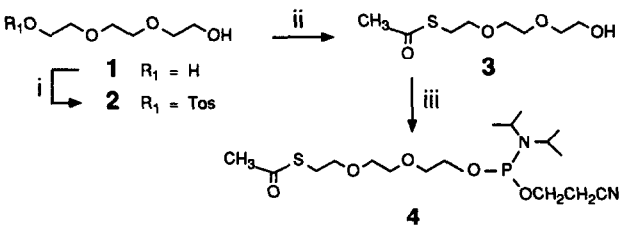
Scheme 1. Pyridyldisulfides can act as precursor for thiolated compounds and can be subsequently oxidized to symmetrical disulfides or reacted with *e.g.* maleimides to form thioethers. Alternatively, pyridyldisulfides can be coupled directly with free thiols to give asymmetrical disulfides.

Ideally, one would like to introduce a thiopyridyl containing thiol function already during solid-phase DNA synthesis. However, pyridyldisulfides are not compatible with the standard phosphoramidite chemistry, since the disulfide linkage will be inevitably reduced by the action of the amidite phosphine. We reasoned that an elegant way to circumvent this problem would be the introduction of an intermediate acetyl-protected thiol linker at the solid support bound oligonucleotide. In this way the formation of the pyridyl-disulfide can be postponed to the deprotection step, as we anticipated that saponification of the thioacetyl function in the presence of DTDP should lead to the *in situ* generation of the desired 5'-thiol function.

In order to suppress efficiently the formation of symmetrical disulfides, the deprotection should be performed in the presence of a large excess of DTDP and under exclusion of oxygen. Such conditions can be easily created when DTDP is dissolved in deoxygenated methanolic potassium carbonate solution. The latter deprotection reagent was introduced by us for the preparation of DNA fragments containing methyl phosphotriester internucleosidic linkages.[15] The combination of potassium carbonate/methanol and the 2-(acetoxymethyl)benzoyl (AMB) nucleobase-protecting group has been recommended for the synthesis of modified DNA fragments containing labile moieties such as phosphate-methylated sequences.[16] Furthermore, this method should be useful for the preparation of more common DNA analogues (*e.g* methylphosphonates and phosphorothioates) and natural sequences as well.

Preparation of thioacetyl-containing linker phosphoramidite.

The thioacetyl linker amidite **4** was prepared starting from triethyleneglycol (**1**), which was converted into its mono-tosylated derivative **2** (Scheme 2). A thioacetyl function could be easily introduced by treating **2** with potassium thiolacetate. Conversion of the resulting compound **3** into phosphoramidite **4** could be readily effected by treatment with chloro-(2-cyanoethoxy)-*N,N*-diisopropylamino-phosphine.



Scheme 2. Preparation of amidite **4**. Reagents: (i) Tosyl chloride, pyridine; (ii) Potassium thiol-acetate; (iii) Chloro-(2-cyanoethoxy)-*N,N*-diisopropylamino-phosphine, *N,N*-diisopropylethylamine.

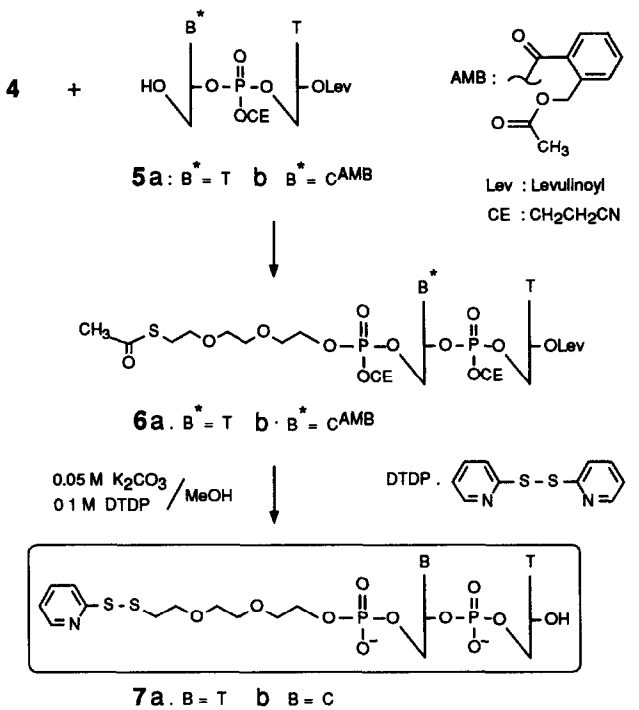
Preparation of pyridyldisulfide-derivatized dinucleotides.

To examine the suitability of our strategy for the synthesis of pyridyldisulfide-derivatized oligonucleotides, amidite **4** was coupled with the 5'-hydroxyl group of the protected dimers **5**. This was accomplished after activation of amidite **4** by 1*H*-tetrazole. The fully protected dinucleotides **6** containing the thioacetyl linker were obtained after *in situ* oxidation of the intermediate phosphite triester with *tert.*-butyl hydro-

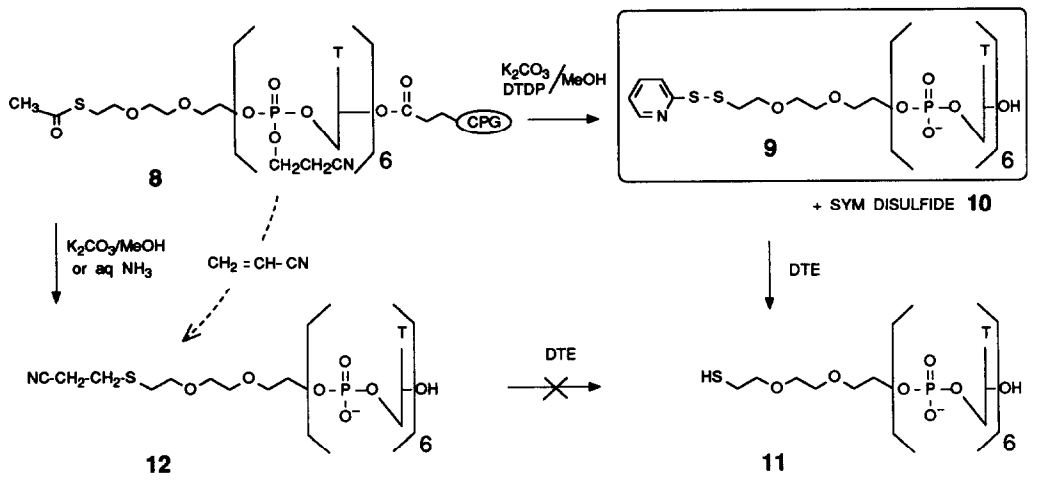
peroxide. Exposure of dimers **6** to 0.05 M potassium carbonate and 0.1 M DTDP in dry oxygen-free methanol, results, apart from removal of the cyanoethyl, AMB and levulinoyl groups, in saponification of the thioacetyl ester and subsequent formation of the pyridyldisulfide linkage (Scheme 3). Work-up was performed under neutral to slightly acidic (pH = 5.5) conditions in order to suppress the formation of symmetrical disulfides. The end products (**7**), which were obtained after lyophilization, contained only minimal amounts of symmetrical disulfide, as gauged by HPLC analysis, while no side-products were observed in the ^{31}P- and ^{1}H NMR spectra. Moreover, the presence of the pyridyl function in dinucleotides **7** was unambiguously ascertained by ^{1}H NMR spectroscopy.

Solid-phase synthesis of pyridyldisulfide-derivatized hexathymidine.

The successful preparation of compounds **7** urged us to apply phosphoramidite **4** in the solid-phase synthesis of the pyridyldisulfide containing hexanucleotide **9** using thymidine-derivatized controlled pore glass (CPG) as the solid support. Chain elongation was performed by employing commercially available cyanoethyl phosphoramidites in a standard protocol. In the final synthetic cycle phosphoramidite **4** was coupled with the fully protected hexamer to afford, after oxidation, immobilized compound **8**. The solid support bound oligonucleotide was treated with potassium carbonate (0.05 M) / DTDP (0.1 M) mixture in oxygen-free dry methanol for 2 1/2 hours at room temperature.



Scheme 3. Preparation of pyridyldisulfide-derivatized dinucleotides **7**



Scheme 4. Deprotection of immobilized hexamer **8** in the presence of DTDP resulting in pyridyldisulfide-derivatized hexamer **9** and a minor amount of symmetrical disulfide **10**. Both can be reduced (DTE) to the thiol containing oligonucleotide **11**. Deprotection without DTDP leads to formation of cyanoethyl adduct **12**

Anion-exchange (MonoQ) HPLC analysis of the end product revealed that the crude mixture contained one main product (**9**) (Figure 1a). The major side-product is, according to its retention time, most likely the highly charged symmetrical disulfide (**10**). Compounds **9** and **10** could be readily separated by Sephadex G-50 column chromatography. The identity of **9** was established by ^{31}P- and ^{1}H NMR spectroscopy. As is evident from the ^{1}H NMR spectrum (Figure 1b), oligonucleotide **9** indeed contains a pyridyl functionality. Both the identity and homogeneity of **9** were further confirmed by reverse phase (RP) HPLC analysis (Figure 1c). Upon treatment with dithioerythritol (DTE) purified **9** was completely reduced to give the faster eluting free thiol containing hexathymidine nucleotide **11** (Figure 1d). DTE reduction of symmetrical disulfide **10** resulted in the same product.

Formation of cyanoethyladducts in the absence of dithiodipyridine.

Since the preparation of **9** was accompanied by the formation of a minor amount of symmetrical disulfide, we also examined the possibility of completely converting **8** into symmetrical disulfide **10** by not including DTDP in the deprotection step. To our surprise, treatment of the fully protected oligonucleotide **8** with methanolic potassium carbonate, followed by oxidation with air, did hardly give any symmetrical disulfide at all, as was found by MonoQ HPLC analysis of the end product (*i.e.* compound **12**). Moreover, exposure of the obtained product to DTE did not lead to formation of the thiolated compound **11**. Same observations were made when **8** was subjected to aqueous ammonia treatment and subsequent oxidation with air. Again this resulted in the formation of compound **12** which is neither the symmetrical disulfide nor the free thiol containing derivative, and which is not susceptible to reduction (DTE) or oxidation (O_2,I_2).

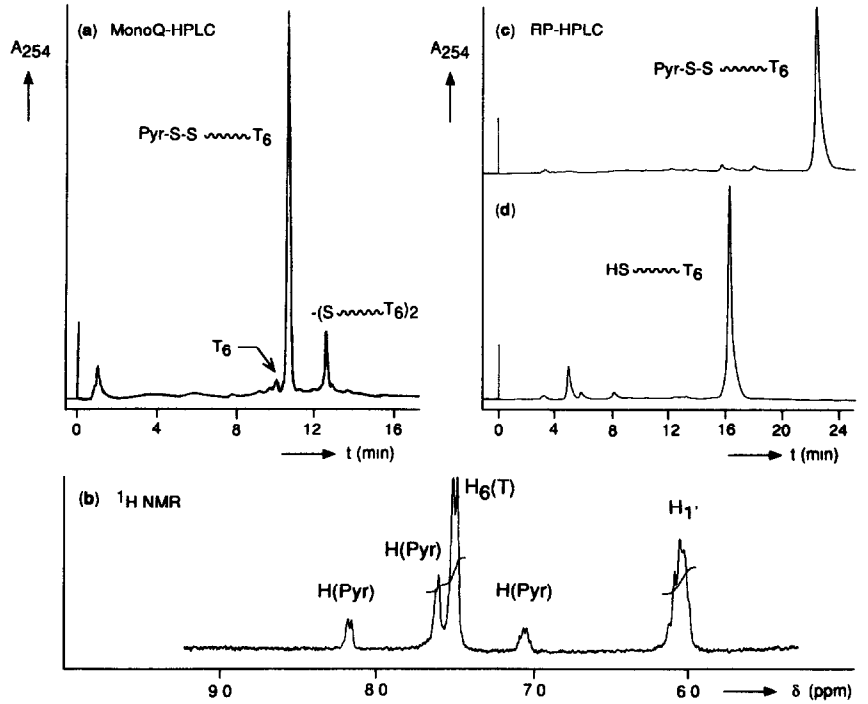


Figure 1. (a) Anion-exchange (MonoQ) HPLC profile of crude oligonucleotide **9**. (b) Part of the ^{1}H NMR spectrum of purified hexamer **9**. (c) Reverse phase HPLC profile of purified **9**. (d) idem, after reduction of **9** with DTE.

In our attempt to find a plausible explanation for the observed phenomena we assumed that, after saponification of the thioacetyl ester, the resulting thiol might have reacted with another species instead of forming a symmetrical disulfide. The most likely candidate for such a side-reaction would be acrylonitrile which is released upon deprotection of the cyanoethyl phosphotriesters. A first indication that acrylonitrile might play a role was obtained after treatment of linker compound **3** with potassium carbonate/methanol in the presence of acrylonitrile. Instead of symmetrical disulfide, the cyanoethyl adduct was formed *via* Michael addition of the liberated thiol function. More direct evidence for occurrence of a Michael addition on acrylonitrile was obtained from characterization of purified **12** by NMR spectroscopy. In the 2.90-2.80 ppm range three clearly distinguishable triplets are found resulting from the methylene groups adjacent to sulfur and cyano function respectively.

Summarizing, direct conversion of thioacetyl-protected oligonucleotides into symmetrical disulfides by simply saponifying the acetyl ester and oxidizing the free thiol, turns out to be impossible in those cases when cyanoethyl-protected phosphate moieties are present. Hence, in one way or another precautions should be taken to prevent the formation of cyanoethyl adducts. The use of DTDP to give thiopyridyl-protected thiol linkers is a convenient prevention, as has been illustrated for the synthesis of oligonucleotide **9**. Since

in the preparation of **9** no cyanoethyl adduct was detected, it may be assumed that in our approach DTDP traps the free thiol groups effectively before addition on acrylonitrile can occur.

Solid-phase synthesis of pyridyldisulfide-derivatized oligomers using AMB base-protected phosphoramidites.

In order to widen the scope of our strategy, we also wished to prepare pyridyldisulfide-derivatized oligonucleotides containing heterosequences. In earlier work we have shown that the 2-(acetoxymethyl)-benzoyl (AMB) group is well suitable for nucleobase protection in the solid-phase synthesis of labile DNA modifications (such as methyl phosphotriesters), in particular when potassium carbonate in methanol is used as deprotection reagent.[15,16] On the other hand, the AMB protective group may also facilitate the preparation of natural DNA sequences. Therefore, the AMB base-protected cyanoethyl phosphoramidite of 5'-dimethoxytrityl-2'-deoxyadenosine (**13**) was utilized in the automated synthesis (1 μmol-scale) of heterooligomer **14**. In analogy with the synthesis of oligonucleotide **9**, a thioacetyl linker was incorporated by applying phosphoramidite **4**. Deprotection of the fully protected oligonucleotide was carried out in 0.05 M potassium carbonate/methanol for 4 hours at room temperature in the presence of DTDP (0.1 M). As expected, this procedure affords pyridyldisulfide-derivatized oligonucleotide **14** as major product, accompanied by some smaller fragments and a minor amount of symmetrical disulfide, as was demonstrated by MonoQ- and RP HPLC analysis (see Figure 2a and 2b).

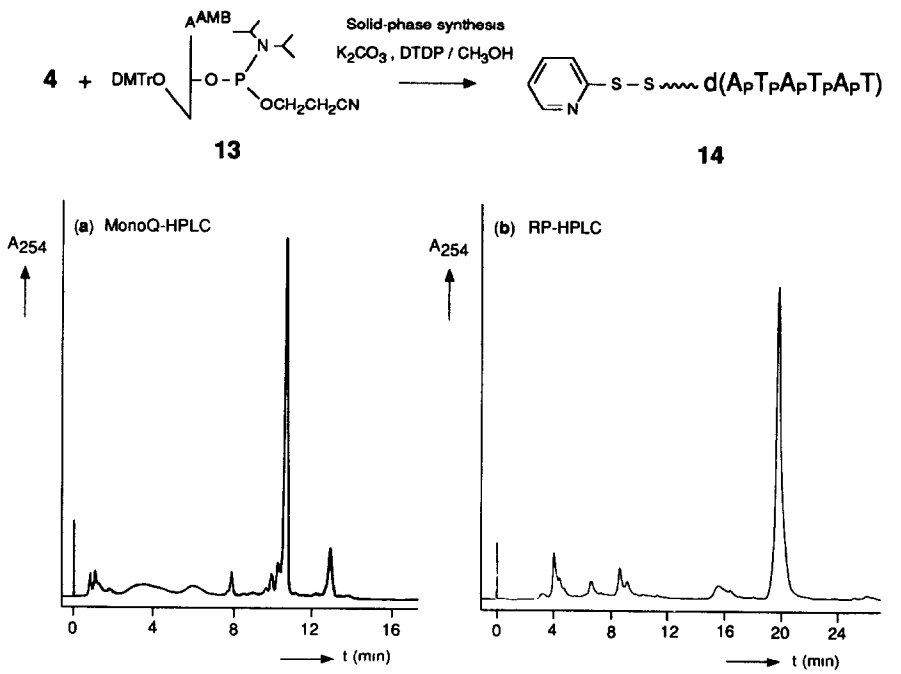


Figure 2. (a) Anion-exchange (MonoQ) and (b) reverse phase HPLC profiles of crude pyridyldisulfide-derivatized oligonucleotide **14** after solid-phase synthesis using thioacetyl linker amidite **4** and AMB-protected amidite **13**. Deprotection was performed with K_2CO_3/DTDP in methanol.

In principle, our strategy is also applicable when the conventional base-protecting groups (benzoyl for A and C, isobutyryl for G) are applied. In that case, instead of potassium carbonate/methanol other deprotecting reagents should be used *e.g.* ammonia or ammonia/methanol usually at elevated temperature. In our experience, however, the ammonia deprotection of thioacetyl-protected oligonucleotides in the presence of DTDP results in a less favorable ratio between pyridyldisulfide and symmetrical disulfide.

Fortunately, several other strategies can be formulated to prevent interference of acrylonitrile in the deprotection procedure. A suitable option might be deprotection of the oligonucleotide in the presence of a large excess of DTE or DTT, which will not only keep the thiolated oligonucleotide in its reduced form, but will also act as scavenger for acrylonitrile. Alternatively, one might consider to first selectively remove the cyanoethyl protecting groups by treating the solid support bound oligonucleotide with triethylamine or diisopropylamine. Subsequently, the oligonucleotide can be cleaved from the support and simultaneously be further deprotected by applying an appropriate reagent (K_2CO_3/CH_3OH, NH_3/CH_3OH, aq. NH_3). Dependent on the procedure used, either free thiols (with DTE) can be obtained, pyridyldisulfides (in the presence of DTDP) or symmetrical disulfides (after air-oxidation). Further research will be directed towards those alternative procedures.

Concluding remarks

The thioacetyl linker amidite described in this paper allows a simple and efficient preparation of pyridyldisulfide-derivatized oligodeoxynucleotides. In comparison with common methods, our strategy does not require modification of solid supports or extensive post-synthetic deprotection procedures. The major advantage of the method described here is that already during DNA deprotection the required pyridyldisulfides are formed through the action of dithiodipyridine. The desired oligomers and the major side-products of this procedure, *i.e.* the symmetrical disulfides, can be easily separated by conventional DNA purification methods such as gel filtration or HPLC (anion-exchange or reverse phase) chromatography. It has been demonstrated that exclusion of DTDP in the deprotection procedure leads to the formation of Michael adducts with acrylonitrile, resulting from deprotection of the cyanoethyl phosphotriesters. Several options have been proposed to prevent concomitant acrylonitrile addition during oligonucleotide deprotection in the absence of DTDP.

Experimental

Abbreviations: AMB: 2-(acetoxymethyl)benzoyl, CE: 2-cyanoethyl, CPG: controlled pore-glass, DTDP: 2,2'-dithiodipyridine, DTE: dithioerythritol, DTT: dithiothreitol, Lev: levulinoyl, RP: reverse phase, Pyr: pyridyl, TEAA: triethylammonium acetate.

General methods and materials

Anhydrous dichloromethane was obtained by distillation from P_2O_5 and stored on basic alumina. Pyridine was dried by heating with CaH_2 and distilled. *N,N*-Diisopropylethylamine was distilled from KOH pellets. Methanol used in deprotection procedures was dried by refluxing on magnesium turnings (5 g/L) for 2

hours and then distilled and stored over molecular sieves (3Å). Anhydrous acetonitrile used to prepare the amidite solutions was purchased from Applied Biosystems (DNA synthesis grade). All other solvents were of pure analysis quality and used without further purification.

Triethylene glycol (Janssen), p-toluenesulphonyl chloride (Janssen), potassium thiolacetate (Aldrich), 2,2'-dithiodipyridine (Aldrithiol™, Aldrich), 1,4-dithioerythritol (Janssen), 1*H*-tetrazole (Janssen), *tert.*-butyl hydroperoxide (80% in di-*tert.*-butylperoxide, Merck) were used as received. Unless otherwise stated, reactions were performed at ambient temperature and under an atmosphere of dry nitrogen.

For thin layer chromatography (TLC) Merck-Fertigplaten (Kieselgel 60, F 254) were used and compounds were detected by spraying with sulphuric acid/ethanol (1:9 v/v).

Nuclear magnetic resonance (NMR) spectra were obtained on either a Bruker WM 200 or a Bruker WM 360 spectrometer. ^{1}H NMR chemical shifts (δ) in organic solvents are reported in ppm relative to tetramethyl silane. For proton spectra in aqueous solution (D_2O) the residual HDO peak was set at 4.80 ppm. ^{31}P NMR data are expressed relative to external 85% H_3PO_4.

The potassium carbonate/DTDP deprotection mixture was prepared as follows. Anhydrous potassium carbonate (dried on P_2O_5 for 72 h at 50°C) was added to degassed dry methanol and dissolved by sonication under slight vacuum to give a 0.05 M solution. This solution was added under nitrogen to DTDP, dried overnight *in vacuo*, to a final concentration of 0.1 M of DTDP.

Triethylammonium acetate buffer (1.0 M) was prepared by adding 96% acetic acid to 1 mole of triethylamine in water (500 mL) to a pH of 5.5 and adjusting the volume to 1000 mL.

High performance liquid chromatography (HPLC) (anion-exchange and reverse phase) was conducted on a Waters 600E (system controller) single pump gradient system. A Waters model 484 variable wavelength UV-detector was used for the detection of oligonucleotides at 254 nm.

Analytical anion-exchange HPLC was executed on a Pharmacia MonoQ HR 5/5 column at a flow rate of 1.0 mL/min, using buffersystem **I** (for compounds **9-12** and **14**) or **II** (for compounds **7** and **16**). System **I**: Buffer A 0.02 M NaH_2PO_4 (pH = 5.5, 25% acetonitrile v/v), buffer B 0.02 M NaH_2PO_4 and 2.0 M NaCl (pH = 5.5, 25% acetonitrile v/v); System **II**: Buffer A 0.001M NaH_2PO_4 (pH = 5.5, 20% acetonitrile v/v), buffer B 0.001 M NaH_2PO_4 and 0.5 M NaCl (pH = 5.5, 20% acetonitrile v/v). Gradients applied are specified for the individual compounds.

Reverse phase HPLC analysis was performed on a Supelco C18 (5μ) column (25 cm x 4.6 mm) at a flow rate of 1.0 mL/min using following buffers (gradient: see individual experiments): Buffer A 0.1 M TEAA (pH = 5.5), buffer B CH_3CN/0.1 M TEAA (pH = 5.5) (9:1 v/v).

2-(2-(2-(p-Toluenesulphonyl)ethoxy)ethoxy) ethanol (2).

To a solution of triethylene glycol (**1**) (60 g, 0.4 mol), dried by evaporation (twice) with anhydrous pyridine, in anhydrous dichloromethane (400 mL) and anhydrous pyridine (6.5 mL) was added p-toluenesulphonyl chloride (7.5 g, 40 mmol). The solution was stirred for 16 hours at room temperature and, subsequently, concentrated under reduced pressure. Residual pyridine was removed by coevaporation with toluene (three times) using an oil-pump. The residue was dissolved in ethyl acetate (250 mL) and washed with aq. NaCl solution (3 x 200 mL). The aqueous phase was extracted with ethyl acetate (2 x 100 mL) and the combined organic layers were dried ($MgSO_4$) and evaporated to dryness to afford crude **2** (10.5 g). The product was further purified by silica gel column chromatography using a gradient of methanol (0→50 vol%) in ethyl acetate as eluent, affording pure **2** as a clear oil (9.3 g, 75% with respect to chloride added). R_f (ethyl acetate/methanol 95:5 v/v): 0.59. ^{1}H NMR ($CDCl_3$): δ 7.80 (2H,d,arom. Tos), 7.35 (2H,d,arom. Tos), 4.18 (2H,t,CH_2-OTos), 3.71 (2H,t,CH_2-OH), 3.60 (8H,m,4×CH_2-O), 2.45 (3H,s,CH_3 Tos).

2-(2-(2-(Thioacetyl)ethoxy)ethoxy) ethanol (3).
Compound **2** (2.5 g, 8.2 mmol) was dissolved in acetone (100 mL) and potassium thiolacetate (2.0 g, 17 mmol) was added. The red-colored solution was refluxed for 2.5 hours. A white precipitate was formed which was filtered off and washed with acetone (50 mL) and ethyl acetate (25 mL). The filtrate was concentrated under reduced pressure and the residue was applied on a silica gel column and eluted with a gradient of methanol (0→10 vol%) in ethyl acetate, yielding a yellow-colored oil (1.66 g). In order to remove the colored component, the product was further purified by column chromatography on silica gel using ethyl acetate/heptane (8:2 v/v) as eluent. This afforded pure **3** as a clear oil (1.63 g, 96%).
R_f (ethyl acetate/heptane 8:2 v/v): 0.44. ^{1}H NMR ($CDCl_3$): δ 3.73 (2H,m,CH_2-OH), 3.70-3.55 (8H,m,4×CH_2-O), 3.11 (2H,t,CH_2-S), 2.35 (3H,s,CH_3 Ac).

2-Cyanoethyl-(2-(2-(2-(thioacetyl)ethoxy)ethoxy)ethyl)-*N,N*-diisopropylphosphoramidite (4).
Compound **3** (0.6 g, 2.9 mmol), dried overnight *in vacuo*, was dissolved in anhydrous dichloromethane (5.0 mL) and *N,N*-diisopropylethylamine (1.5 mL, 9.0 mmol) was added. The solution was cooled in an ice-water bath and chloro-(2-cyanoethoxy)-*N,N*-diisopropylamino-phosphine (1.0 mL, 4.4 mmol) was added in a dropwise manner. After stirring for 30 minutes, TLC-analysis revealed complete conversion into phosphoramidite **4** (R_f (ethyl acetate/heptane/triethylamine 10:9:1 v/v/v): 0.75). The reaction mixture was diluted with ethyl acetate (60 mL) and washed with ice-cold saturated aq. NaCl (3 x 50 mL). The aqueous phase was extracted with ethyl acetate (2 x 30 mL) and the combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. The resulting oil was purified by flash chromatography on silica gel, using heptane /ethyl acetate/triethylamine (80:15:5 v/v/v) as eluent, affording pure **4** as a clear oil (0.96 g, 82%).
R_f (heptane/ethyl acetate/triethylamine 80:15:5 v/v/v): 0.27
^{31}P NMR ($CDCl_3$): δ 148.8 ppm. ^{1}H NMR ($CDCl_3$): δ 3.85 (2H,m,CH iPr), 3.75-3.55 (12H,m,5×CH_2-O and CH_2 CE), 3.09 (2H,t,CH_2-S), 2.66 (2H,t,CH_2 CE), 2.34 (3H,s,CH_3 Ac), 1.19 (12H,dd,CH_3 iPr).

Preparation of dimer 6a.
To a solution of compound **5a** [15] (116 mg, 0.166 mmol) in dry dichloromethane (2.5 mL) was added a 0.5 M solution of 1*H*-tetrazole in anhydrous acetonitrile (2.0 mL, 1.0 mmol). Phosphoramidite **4** was added as a 0.1 M solution in anhydrous acetonitrile (3.0 mL, 0.30 mmol). After 30 min at room temperature, TLC analysis (CH_2Cl_2/CH_3OH 9:1 v/v) showed that dimer **5a** was still present. Thus, additional portions of 1*H*-tetrazole (0.5 mmol) and phosphoramidite **4** (0.2 mmol) in dry acetonitrile were added and the reaction mixture was stirred for another 15 min. Oxidation of the intermediate phosphite triester was accomplished by the addition of *tert.*-butyl hydroperoxide (0.6 mL). After stirring for 10 min, the reaction mixture was diluted with dichloromethane (40 mL). The organic phase was washed with with saturated aq. NaCl (2 x 50 mL), and the aqueous layers were extracted with ethyl acetate (2 x 25 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to dryness. Pure compound **6a** was obtained after silica gel column chromatography using a gradient of methanol (10→20 vol%) in ethyl acetate. Yield: 147 mg, 87%.
R_f (CH_2Cl_2/CH_3OH 9:1 v/v): 0.54.
^{31}P NMR ($CDCl_3$): δ -1.20, -1.63, -2.30 and -2.46 ppm. ^{1}H NMR ($CDCl_3$) of a mixture of diastereoisomers: δ 7.37 (2H,m,2×H_6(T)), 6.28 (2H,m,2×$H_{1'}$), 5.30 (1H,m,$H_{3'}$), 5.17 (1H,m,$H_{3'}$), 4.45-4.15 (12H,m,2×-$H_{4'}$/2×CH_2 CE/CH_2O-P and 2×$H_{5'}$/$H_{5''}$), 3.75-3.50 (8H,m,4×CH_2-O), 3.07 (2H,t,CH_2-S), 2.90-2.15 (4H,m,-2×$H_{2'}$/$H_{2''}$), 2.83 and 2.80 (6H,2×t,2×CH_2 CE and CH_2 Lev), 2.58 (2H,t,CH_2 Lev), 2.34 (3H,s,CH_3 Ac), 2.20 (3H,s,CH_3 Lev), 1.94 (6H,m,2×CH_3(T)).

Preparation of dimer 6b.

Dimer **5b** (130 mg, 0.152 mmol), the preparation of which has been described previously[16], was dissolved in anhydrous dichloromethane (2.5 mL). To this solution were added 1*H*-tetrazole (1.8 mL of a 0.5 M stock solution in dry acetonitrile, 0.9 mmol) and phosphoramidite **4** (2.5 mL of a 0.1 M solution in dry acetonitrile, 0.25 mmol). Since TLC-monitoring (ethyl acetate/methanol 85:15 v/v) of the reaction revealed the presence of starting dimer **5b**, an additional amount of phosphoramidite **4** was added (1.0 mL, 0.10 mmol). The solution was cooled (ice-water bath) and *tert.*-butyl hydroperoxide was added (0.25 mL). Oxidation was allowed to proceed for 30 min at 0°C. The reaction mixture was diluted with ethyl acetate (25 mL) and washed with saturated aq. $NaHCO_3$ (2 x 25 mL) and saturated aq. NaCl (25 mL). The aqueous layers were back-extracted with ethyl acetate (2 x 15 mL). The combined organic layers were dried on $MgSO_4$ and concentrated under reduced pressure. Crude dimer **6b** was chromatographed on a silica gel column using dichloromethane/ethyl acetate/methanol (5:4:1 v/v/v) as eluent. This afforded pure compound **6b** as a white solid (146 mg, 83%). R_f (ethyl acetate/CH_3OH 85:15 v/v): 0.28, R_f (CH_2Cl_2/ethyl acetate/-CH_3OH 5:4:1 v/v/v): 0.27.

^{31}P NMR ($CDCl_3$): δ -1.08, -1.44, -2.33 and -2.58 ppm. ^1H NMR ($CDCl_3$) of a mixture of diastereoisomers: δ 8.16 (1H,d,H_6(C)), 7.68 (1H,m,arom. AMB), 7.60-7.40 (3H,m,arom. AMB), 7.35 (1H,s,H_6(T)), 6.25 (2H,m,2×$H_{1'}$), 5.36 (2H,s,CH_2 AMB), 5.30 (1H,m,$H_{3'}$), 5.18 (1H,m,$H_{3'}$), 4.50-4.15 (12H,m,2×$H_{4'}$/2×CH_2 CE/CH_2-O-P and 2×$H_{5'}$/$H_{5''}$), 3.80-3.55 (8H,m,4×CH_2-O), 3.06 (2H,t,CH_2-S), 3.00-2.00 (4H,m,2×$H_{2'}$/$H_{2''}$), 2.83 and 2.79 (6H,2×t,2×CH_2 CE and CH_2 Lev), 2.59 (2H,t,CH_2 Lev), 2.33 (3H,s,CH_3 Ac), 2.20 (3H,s,CH_3 Lev), 2,12 (3H,s,CH_3 AMB), 1.93 (3H,s,CH_3(T)).

General procedure for the preparation of thioacetyl-derivatized dimers 7.

Thioacetyl-containing dimer **6** (20 μmol) was dissolved in a freshly prepared, degassed solution of potassium carbonate (0.05 M) and DTDP (0.1 M) in anhydrous methanol (5 mL; see general methods). At the time of complete deprotection of Lev- and/or AMB groups (1 h for **6a**, 6 h for **6b**), the mixture was neutralized carefully by the addition of 5 vol% acetic acid to give a final pH of 5.5. Water was added to a total volume of 5 mL. Methanol was evaporated under reduced pressure, resulting in the formation of a white precipitate (DTDP). Excess DTDP was removed by washing the aqueous phase with diethyl ether (3 x 5 mL). The aqueous layer was concentrated to a small volume (1 mL) and desalted by Sephadex G-10 chromatography (1 cm x 50 cm) using 0.05 M TEAA as eluent. The appropriate fractions were pooled and lyophilized.

Retention times MonoQ (Buffersystem **II**; gradient: 0-2 min 100% A, 2-22 min 0-75% B). Rt (**7a**): 11.8 min, Rt (corresponding sym.disulf): 16.1 min. Rt (**7b**): 11.0 min, Rt (corresponding sym.disulf): 16.3 min.

^{31}P NMR ($CDCl_3$) (**7a**): δ 0.68 and -0.62 ppm. ^1H NMR ($CDCl_3$) (**7a**) of a mixture of diastereoisomers: δ 8.35 (1H,d,Pyr), 7.80 (2H,m,2×H_6(T)), 7.69 (2H,d,Pyr), 7.25 (1H,m,Pyr), 6.29 (2H,m,2×$H_{1'}$), 4.91 (1H,m,-$H_{3'}$), 4.81 (1H,m,$H_{3'}$), 4.58 (1H,m,$H_{4'}$), 4.36 (1H,m,$H_{4'}$), 4.20-4.00 (6H,m,CH_2O-P and 2×$H_{5'}$/$H_{5''}$), 3.80-3.50 (8H,m,4×CH_2-O), 2 98 (2H,t,CH_2-S), 2.52 (1H,m,$H_{2'}$), 2.35 (3H,m,2×$H_{2'}$/$H_{2''}$), 1.88 (6H,m,CH_3(T)).

^{31}P NMR ($CDCl_3$) (**7b**): δ 0.69 and -0.67 ppm. ^1H NMR ($CDCl_3$) (**7b**) of a mixture of diastereoisomers: δ 8.33 (1H,d,Pyr.), 7.84 (1H,d,H_6(C)), 7.80 (2H,d,Pyr), 7.69 (1H,s,H_6(T)), 7.22 (1H,m,Pyr), 6.27 (2H,m,-2×$H_{1'}$), 5.99 (1H,d,H_5(C)), 4.85 (1H,m,$H_{3'}$), 4.78 (1H,m,$H_{3'}$), 4.57 (1H,m,$H_{4'}$), 4.34 (1H,m,$H_{4'}$), 4.15-3.90 (6H,m, CH_2O-P and 2×$H_{5'}$/$H_{5''}$), 3.75-3.45 (8H,m,4×CH_2-O), 2.96 (2H,t,CH_2-S), 2.55 (1H,m,$H_{2'}$), 2.35-2.10 (3H,m,2×$H_{2'}$/$H_{2''}$), 1.87 (3H,s,CH_3(T)).

Solid-phase synthesis of immobilized fully protected oligomer 8.
Oligonucleotide **8** was prepared on an Applied Biosystems DNA synthesizer (Model 381A), using phosphoramidite **4** and the commercially available 5'-*O*-(4,4'-dimethoxytrityl)-thymidine 3'-*O*-(2-cyanoethyl)-*N,N*-diisopropylphosphoramidite (ABI). The assembly of the thymidine nucleotides was performed on a 10 μmol-scale (thymidine-loaded CPG, ABI) according to the standard protocol.[17] After delivery of the thymidine amidite solution, premixed with tetrazole, to the column a "wait" period of 1 min was included instead of the standard 15 seconds. A 0.1 M solution of phosphoramidite **4** in anhydrous acetonitrile was prepared and filtered through a 0.45 μm teflon filter prior to use. In comparison with the standard procedure, this amidite solution was passed through the column for an additional 2 min, and, subsequently, the coupling raction was allowed to proceed for 5 min. After the standard oxidation step, the solid support was thoroughly washed with acetonitrile (the capping procedure was not included in this cycle). The solid support was dried *in vacuo* before performance of the deprotection procedure.

Preparation of pyridyldisulfide-derivatized oligonucleotide 9.
The solid support, containing immobilized oligonucleotide **8** (10 μmol), was removed from the column under nitrogen and treated with a solution of DTDP (0.1 M) and potassium carbonate (0.05 M) in anhydrous methanol (15 mL; see general methods). After a reaction time of 2 1/2 hours at room temperature, the solution was neutralized by carefully adding 5 vol% acetic acid in water to a final pH of 5.5. The solid support was filtered off and washed with methanol/water (1:1 v/v, 5 mL) and water (5 mL). The filtrate was concentrated under reduced pressure to a volume of 10 mL. The white precipitate (excess DTDP) was removed by washing several times with diethyl ether (5 x 10 mL). The aqueous phase was concentrated to a small volume (2.5 mL) and chromatographed on a Sephadex G-50 column (90 x 5 cm^2) eluted with 0.05 M TEAA (pH = 5.5) at a flow rate of 15 mL/h. The first UV-absorbing peak (elution time: 15.7 h) contained symmetrical disulfide **10**. Fractions corresponding with the main peak (elution time: 21.9 h), containing oligonucleotide **9**, were pooled and concentrated to a small volume. After passage through a column of Dowex 50W X8 cation-exchange resin (Na^+ form), the oligonucleotide solution was lyophilized to give oligonucleotide **9** as a white solid (9.3 mg, 4.4 μmol).
For NMR measurements the oligonucleotide was lyophilized twice from D_2O (99.75%) and dissolved in 500 μL of D_2O (99.95%).
Retention times MonoQ (Buffersystem I; gradient: 0-30 min 0-96% B): Rt (T_6): 10.1 min, Rt (**9**): 10.7 min, Rt (**10**) 12.6 min. Retention times RP (Gradient: 0-5 min 10-12% B, 5-25 min 12-26 %B): Rt (T_6): 12.4 min, Rt (**9**): 22.5 min, Rt (**10**): 18.3 min.
^{31}P NMR (D_2O): δ 0.67 and -0.68 ppm (ratio 1:5). ^1H NMR (D_2O) : δ 8.33 (1H,d,Pyr), 7.77 (2H,m,Pyr), 7.67 (6H,m,6×H_6(T)), 7.23 (1H,m,Pyr), 6.30 (1H,dd,$H_{1'}$), 6.25 (5H,m,5×$H_{1'}$), 4.89 (1H,m,$H_{3'}$), 4.78 (2H,m,2×$H_{3'}$), 4.58 (1H,m,$H_{3'}$), 4.36-4.29 (6H,m,6×$H_{4'}$), 4.18-4.04 (12H,m,6×$H_{5'}$/$H_{5''}$), 4.00 (2H,m,CH_2O-P), 3.70 (4H,m,2×CH_2-O), 3.62 (2H,m,CH_2-O), 3.53 (2H,m,CH_2-O), 2.97 (2H,t,CH_2-S), 2.56-2.20 (12H,m,-12×$H_{2'}$/$H_{2''}$), 1.87 (18H,m,6×CH_3(T)).

Deprotection of oligomer 8 in potassium carbonate/methanol affording hexamer 12.
To solid support with immobilized oligonucleotide **8** (4 μmol) was added a freshly prepared 0.05 M solution of potassium carbonate in dry methanol (5 mL). Deprotection was allowed to proceed for 2 1/2 hours at room temperature and during the last 30 minutes air was bubbled through the solution. After neutralization of the solution with 5 vol% acetic acid, the solid support was removed by filtration and washed with methanol/water (1:1 v/v; 2 mL) and water (4 mL). The filtrate was concentrated to a small

volume and desalted on a Sephadex G-25 column (45 cm x 1 cm) at a flow rate of 15 mL/h using 0.05 M TEAA (pH = 5.5) as eluent. The appropriate fractions were pooled and further treated as described for oligonucleotide **9**. This afforded 5.6 mg (2.7 μmol) of compound **12**.

Retention times MonoQ (Buffersystem I; gradient: 0-2 min 100% A, 2-32 min 0-100% B): Rt (**12**) 11.4 min, Rt (**10**) 13.3 min. Retention times RP (Gradient: 0-5 min 10-12% B, 5-25 min 12-26% B): Rt (**12**) 17.5 min, Rt (**10**) 18.3 min.

^{31}P NMR (D_2O): δ 0.59 and -0.68 ppm (ratio 1:5). ^{1}H NMR (D_2O) : δ 7.72 (1H,d,H_6(T)), 7.69 (1H,d,-H_6(T)), 7.67 (1H,s,H_6(T)), 7.65 (3H,m,3×H_6(T)), 6.31 (1H,dd,$H_{1'}$), 6.27 (5H,m,5×$H_{1'}$), 4.89 (1H,m,$H_{3'}$), 4.78 (2H,m,2×$H_{3'}$), 4.59 (1H,m,$H_{3'}$), 4.36 (1H,m,$H_{4'}$), 4.31 (5H,m,5×$H_{4'}$), 4.19-4.05 (12H,m,6×$H_{5'}$/$H_{5''}$), 4.00 (2H,m,CH_2O-P), 3.73-3.66 (8H,m,4×CH_2-O), 2.85, 2.84 and 2.81 (6H,3×t,2×CH_2-S and CH_2-CN), 2.57-2.46 (6H,m,6×$H_{2'}$), 2.40-2.25 (6H,m,6×$H_{2''}$), 1.87 (18H,m,6×CH_3(T)).

Verification of the identity of hexamers 9 and 12 using reverse phase HPLC.

Reduction of hexamer 9 with DTE (Figure 1d).

An analytical amount of pyridyldisulfide-containing oligonucleotide **9** in 0.1 M phosphate buffer (pH = 8.0)/methanol (2:1 v/v) (250 μL) was treated with DTE (1 mg). Samples were taken and analyzed by RP-HPLC. Gradient: 0-5 min 10-12% B, 5-25 min 12-26% B. Rt (pyridyldisulfide **9**) 22.5 min, Rt (free thiol **11**) 16.4 min.

Reduction of hexamer 9 with tributylphosphine and formation of cyanoethyladduct 12.

Oligonucleotide **9** (analytical amount) was dissolved in a degassed mixture of 0.1 M phosphate buffer (pH = 8.0) and methanol (2:1 v/v) (500 μL). To this solution were added small portions (5μL) of 0.005 M tributylphosphine in degassed 2-propanol.[18] RP-HPLC monitoring of the reduction revealed the presence of pyridyldisulfide **9** and symmetrical disulfide **10** until an equimolar amount of tributylphosphine had been added resulting in formation of thiolated derivative **11**. At that stage, the solution was divided into two equal portions. One portion was allowed to oxidize on the air. RP-HPLC analysis after one day showed complete conversion of **11** into symmetrical disulfide **10**. To the other portion were added small amounts (5 μL) of a 1 vol% solution of acrylonitrile in 2-propanol. After each addition a sample was taken and analyzed by RP-HPLC. This, finally, resulted in the formation of a single product, showing identical behavior on MonoQ- and RP-HPLC as oligonucleotide **12**. The addition of larger amounts of acrylonitrile gave several products, probably due to polymerization on the linker chain.

Gradient: 0-20 min 12-26% B. Rt (pyridyldisulfide **9**): 17.9 min, Rt (sym. disulfide **10**): 13.9 min, Rt (free thiol **11**): 12.0 min, Rt (cyanoethyladduct **12**): 13.5 min.

Preparation of phosphoramidite 13.

This compound was synthesized by phosphitylation of 5'-*O*-(4,4'-dimethoxytrityl)-6-*N*-(2-(acetoxymethyl)-benzoyl)-2'-deoxyadenosine[16] using the procedure described for phosphoramidite **4**.

Solid-phase synthesis of pyridyldisulfide-derivatized oligomer 14.

Oligonucleotide **14** was prepared on a 1 μmol-scale[17] using phosphoramidite **13** as 0.1 M solution in acetonitrile in combination with commercially available thymidine cyanoethyl phosphoramidite (ABI). The delivery of thioacetyl-containing linker phosphoramidite **4** in the final synthetic cycle was extended for 25 seconds, followed by a coupling time of 5 min. The capping procedure was omitted in this cycle. The solid supports were dried *in vacuo* and treated with potassium carbonate (0.05M) / DTDP (0.1 M) solution in degassed dry methanol (2.5 mL; see general methods) for 4 hours at room temperature. Work-up was

performed as described for oligonucleotide **9**. Compound **14** was purified by Sephadex G-50 chromatography (65 cm x 1 cm) using 0.05 M TEAA as eluent (flow rate 10 mL/h).
Rt 10.8 min (MonoQ; buffersystem I; gradient: 0-30 min 0-96% B), Rt 19.6 min (RP; gradient: 0-5 min 10-12% B, 5-25 min 12-26% B).

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