Synthesis and selective cleavage of an oligodeoxynucleotide containing a bridged internucleotide 5'-phosphorothioate linkage

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ABSTRACT

A self complementary oligodeoxynucleotide dodecamer containing an achiral bridged 5'-phosphorothioate linkage 3'-O-P-S-5' has been prepared using the solid phase phosphoramidite procedure. For the incorporation of the phosphorothioate link we have used a 5'-(S-trityl)-mercapto-5'-deoxythymidine-3'-phosphoramidite. After coupling this building block the S-trityl group was removed by silver ions. The free thiol moiety was then coupled with a standard phosphoramidite in the presence of tetrazole. After oxidation of the 5'-phosphorothioate with iodine/water the synthesis was continued with standard building blocks up to the desired dodecamer. This backbone modified dodecamer can be cleaved selectively and quantitatively at the P-S bond by silver or mercuric ions under very mild conditions.

INTRODUCTION

The synthesis of oligo(deoxy)nucleotides (ODNs) containing internucleotide modifications at phosphorus is of increasing interest. These ODNs could act as modulators of gene expression and have shown antiviral activity.¹ Furthermore they are useful for studies on the interactions of nucleic acids with DNA-processing enzymes, repressors or for the chemoselective manipulation of DNA. A wide range of prominent candidates for this purpose are already described (FIGURE 1, 1-7).² Bridged analogs like 8-11 have only received little attention.³⁻¹⁰ In analogy to our¹¹⁻¹² and other¹³ work about the preparation of ODNs containing bridging phosphoramidate internucleotide linkages (8-9) we expected that analogs where one of the bridging oxygen atoms is replaced by sulfur (10-11) are also of interest. These compounds are achiral at phosphorus and thus no diastereomers are created during their synthesis. A further advantage is their electronic and steric similarity with the natural congener. Up to now all analogs containing bridged 3'-O-P-S-5' linkages have been prepared via nucleophilic displacement of 5'-O-tosyl- or 5'-iodonucleosides by nucleoside 3'-phosphorothioates.⁵⁻⁸ The aim of this work was to develop a method

for incorporating a bridged 3'-O-P-S-5' internucleotide linkage which can be adapted to automated DNA synthesis via the phosphoramidite chemistry.¹⁴ The solid phase synthesis of an ODN containing a 3'-phosphorothioate linkage (11) using a thymidine-3'-S-phosphorothioamidite was recently described by Cosstick and Vyle.⁹ Due to the greater nucleophilicity of the thiol- contrary to the hydroxyl-group, thionucleosides or nucleosidephosphorothioates may also be involved in the non-enzymatic synthesis of ODNs in prebiotic time. This fact could have contributed to the origin of life.^{15,16} Here we describe the synthesis, purification, characterization and selective cleavage of an ODN containing a 3'-O-P-S-5' linkage.

RESULTS AND DISCUSSION

Synthesis of the monomer building block

The key intermediate useful for the synthesis of ODNs containing a bridged 5'-phosphorothioate linkage is the phosphoramidite **15a,b**. The preparation of this modified thymidine building block is illustrated in FIGURE 2. This compound has been prepared in a modified procedure to the previously described one by Sproat et al.¹⁷ Thus thymidine **12** was reacted with two equivalents of p-toluenesulfonyl chloride in pyridine at r.t. for three hours. The reaction proceeds with satisfactory regioselectivity (70% 5'-tosylate) and the 5'-tosylate **13** could be isolated by selective crystallisation from ethanol in 61 % yield.¹⁸ Therefore it is not necessary to introduce a 3'-OH protecting group. This compound was converted under argon to 5'-(S-trityl)-mercapto-5'-deoxythymidine **14** by a S_N2 reaction with five equivalents sodium tritylthiolate which was prepared in situ from tritylmercaptan and sodium hydroxide in ethanol/water (reflux, 8 hours). After flash chromatographic purification on silica gel and precipitation into cold n-pentane the compound was isolated as a colorless solid in 56 % yield. Subsequent phosphorylation with 2-cyanoethoxybis-(N,N-diisopropylamino)-phosphane in the presence of tetrazole afforded the desired 3'-O-phosphoramidite building block **15a,b**. After purification by flash chromatography and precipitation into cold n-pentane **15a,b** could be isolated as an amorphous colorless powder in 83 % yield.

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Figure 1. Oligodeoxynucleotides with a modified internucleotide phosphate residue.

Figure 2. Scheme for the preparation of the modified thymidine building block 15. Reagents: i, TosCl in pyridine; ii, Ph₃C-SNa in ethanol/water; iii, 2-cyanoethoxy-bis-(N,N-diisopropylamino)-phosphane in CH₃CN/CH₂Cl₂.

Synthesis and purification of the backbone modified ODN 16

To test the possibility of a coupling reaction of a 5'-thiol function with a standard 3'-O-phosphoramidite we tried to prepare a self complementary dodecamer containing a bridged 5'-phosphorothioate linkage (TABLE 1., 16).

When this work was in progress Caruthers et al. described a method whereby dinucleoside phosphorodithioates are synthesized from a dinucleoside phosphoramidite with a mercaptan in the presence of tetrazole followed by oxidation with sulfur.¹⁹

The utility of the modified thymidine building block was demonstrated by the synthesis of 16 (FIGURE 3.). This ODN was synthesized from the heptamer C GAG TCT which was prepared using standard solid phase phosphoramidite chemistry. The 5'-S building block 15a,b was utilized under standard conditions in an automated DNA synthesizer yielding ODN I. The cleavage of the S-trityl group was performed using a 50mM aqueous silver nitrate solution at r.t. for 15 minutes and then the resin was washed with water. This heavy metal cleavage reaction was necessary because the S-trityl function can not be easily cleaved by mild acids which are commonly used to deprotect

Table 1. Synthesized oligodeoxynucleotides and average and overall yields as determined by trityl color quantitation at 498nm

Oligo Number	Synthesized Sequence	Cycle Used	Average Yield	Overall Yield
16	d(AGA C ₅ TC GAG TCT)	I,II	97%	69%
17	d(pAGA C)	I	98%	90%
19	d(TrS-TCG AGT CT)	I	98%	85%
20	d(SH-TCG AGT CT)	I	—	—
21	d(OH-TCG AGT CT)	I	99%	92%

Figure 3. Scheme for the synthesis of the backbone modified dodecamer 16. Reagents: i, 1. 50mM AgNO₃aq, 2. 50mM dithiothreitol; ii, 1. tetrazole in CH₃CN, 2. I₂/water; iii, three standard phosphoramidite reaction cycles.

5'-O-dimethoxytritylnucleotides.²⁰ To ensure that no disulphide formation has occurred the resin was then treated with 50mM dithiothreitol for five minutes. After this time the column was washed with water and dried by several washes with anhydrous acetonitrile. The resulting free thiol moiety from II was then reacted with a tenfold excess of a standard 'C' phosphoramidite (III) in the presence of tetrazole. This reaction was allowed to proceed for 300s at r.t. which leads to the 5'-thiophosphite in 85% yield as determined by trityl color quantitation. The P(III) linkage was then oxidized to the corresponding phosphorothioate by iodine/water (IV). After this step the synthesis was continued under standard conditions up to the desired dodecamer. The two different synthesis cycles are summarized in TABLE 2. Treatment with conc. ammonia removed the protected oligomer from the solid support and the phosphate and aglycon protecting groups were deblocked at 55°C during 16 hours. After evaporation to dryness the crude ODN was purified by preparative polyacrylamide gel electrophoresis (3mm, 16% polyacrylamide/7M urea, 6500Vh). The stability of the P-S bond towards the iodine/water treatment during the four repetitive synthesis cycles is surprising in view of the reported susceptibility against iodine.²¹

Characterization of the backbone modified ODN 16

To make the incorporation of the bridged internucleotide P-S linkage evident we recorded a ³¹P-NMR spectrum (FIGURE 4.). This spectrum shows two resonances located at 0,39 and

Table 2. Synthesis cycles used for oligodeoxynucleotide synthesis (NMI:N-Methylimidazole)

Reagent	Function	Cycle I	Cycle II
3% TCA/CH ₂ Cl ₂	to column	50s	—
50mM AgNO ₃	"	—	20s
	detritylation	1s	900s
50mM DTT	to column	—	20s
	reduction	—	300s
amidite + tetrazole	to column	5+7s	5+7s
	condensation	30s	300s
Ac ₂ O/NMI/THF	to column	20s	20s
	capping	1s	1s
I ₂ /H ₂ O/pyridine	to column	23s	23s
	oxidation	30s	30s

Figure 4. Proton decoupled ³¹P-NMR spectrum of ODN 16 measured in H₂O/D₂O (85:15) at 303K (10 OD₂₆₀ units in 0.6ml, 64008 scans).

18.77ppm corresponding to the phosphate groups and the 5'-S-phosphorothioate respectively. The integrated areas of the NMR peaks gave the correct PO/PS ratio of 10:1.

Selective chemical cleavage of ODN 16

Cleavage of the P-S bond by aqueous silver nitrate or mercuric chloride. To evaluate specific chemical conditions for the cleavage of the P-S internucleotide bond we anticipated that heavy metal ions or iodine may give good results (**FIGURE 5**).^{9,22} First of all we tested the susceptibility of 16 to a 50mM aqueous silver nitrate solution. Therefore one A₂₆₀ unit of the oligomer was treated with 10μl of the Ag⁺ solution. After 15 minutes at room temperature 5μl formamide was added and the solution was loaded onto a polyacrylamide gel to analyze the cleavage reaction. **FIGURE 6a** shows the UV shadowing of the gel together with the backbone modified ODN 16.

Synthesis of reference oligodeoxynucleotides for comparison with the cleavage products. In order to analyze the cleavage products A G A Cp and HS-T C G A G T C T which should result from the cleavage reaction we have synthesized two ODNs for

Figure 5. Selective cleavage of the phosphorothioate linkage of the ODN 16. Reagents: i, 50mM AgNO₃ or HgCl₂; ii, excess dithiothreitol; iii, without dithiothreitol. **Figure 6a.** UV-shadowing of the analytical 16% polyacrylamide gel containing 7M urea for analyzing the AgNO₃ cleavage reaction of ODN 16. Lane 1: bromophenol blue marker, lane 2: 17, lane 3: 16 + AgNO₃, lane 4: 19 + AgNO₃, lane 5: 16 + AgNO₃ + DTT, lane 6: 19 + AgNO₃ + DTT, lane 7: 21, lane 8: 16, lane 9: 19. **b.** UV-shadowing of the analytical 16% polyacrylamide gel containing 7M urea for analyzing the iodine cleavage reaction of ODN 16. Lane 5: 16 + AgNO₃ + DTT, lane 10: 19 + I₂ + DTT, lane 11: 19 + I₂, lane 12: 16 + I₂ + DTT, lane 13: 16 + I₂.

comparison. Because it is difficult to synthesize 3'-phosphorylated ODNs we prepared the 5'-isomer pA G A C (**17**). This compound was synthesized by chemical phosphorylation of the corresponding tetramer with bis-2-cyanoethoxy-N,N-diisopropylamino-phosphane and tetrazole using cycle I (TABLE 2.).²³ After oxidation with iodine/water the oligomer was cleaved from the support and deprotected with conc. ammonia at 55°C during 16 hours. The 5'-thiolate octamer **20** was prepared from the S-trityl containing ODN **19** by deblocking with aqueous 50mM silver nitrate solution for 15 minutes at room temperature.

Analysis of the heavy metal cleavage reactions by polyacrylamide gel electrophoresis. The above two ODNs were analyzed together with the cleavage reaction. FIGURE 6a shows that the sulfur bridged ODN **16** was cleaved selectively into two ODNs (lane 3). As expected the synthesized chemically 5'-phosphorylated tetramer **17** has the same electrophoretic mobility as the 3'-isomer. On the other hand the second cleavage band has a too low electrophoretic mobility compared to the dodecamer **16** in lane 8. Because the electrophoretic shift of this band is almost like a 16mer we argued that this band represents the oxidized 5'-SH ODN which is joined via a disulfide bond (FIGURE 5.). This band has the same electrophoretic mobility as the S-trityl octamer **19** after treatment with 50mM silver nitrate at r.t. for 15 minutes (lane 4). In order to prove the above assumption we tried to reduce the putative disulfide adduct. Therefore the dodecamer **16** was treated firstly with 10 μ l of a 50mM aqueous silver nitrate solution (15min., r.t.) followed by an excess of dithiothreitol. The precipitated silver dithiothreitol complex was removed by centrifugation. To the supernatant was added 5 μ l of formamide and the reaction mixture was analyzed by gel electrophoresis to determine the cleavage products (lane 5). The slower migrating band has the same mobility as the S-trityl octamer which was treated as outlined above (lane 6).

Lane 7 shows the 5'-OH octamer **21** which migrates a little bit faster in the gel compared to the SH-octamer **20**. In lane 9 one can see the ODN **19** which is still SH-protected. When the above cleavage reactions were performed with HgCl₂²⁴ instead of AgNO₃ we got the same results as mentioned above. The above procedure for cleaving the P-S bond achieved by treatment with AgNO₃ or HgCl₂ is in analogy to that described by Connolly and Rider for the deblocking of S-trityl functions from conjugated ODNs.²⁰

Cleavage of the P-S bond with aqueous iodine. As described in the literature for the cleavage of the C-S bond of S-trityl protected cysteine residues using iodine we tried to cleave the P-S bond under similar conditions.^{22,25} Lane 11 in FIGURE 6b shows the cleavage reaction of **19** with 25mM iodine in water/methanol (1:1, v:v) and in lane 10 the same ODN which was treated with iodine followed by an excess of dithiothreitol. In lane 11 one can see only a little of the expected disulfide. As the major products one can see two other bands. One of this bands has a similar electrophoretic mobility compared with the SH-octamer, the other band has a higher mobility in the gel matrix. After addition of dithiothreitol (lane 10) the disulfide was reduced. Lane 13 shows the cleavage reaction of the dodecamer **16** after treatment with 10 μ l of 25mM iodine solution and lane 12 the same reaction after addition of an excess dithiothreitol. The reaction produces four products, the upper band has a similar mobility as the SH-octamer, whereas the band with the highest electrophoretic mobility represents the 3'-phosphorylated tetramer

AGACp. One of the two other bands corresponds with one which is also produced in the cleavage reaction of **19**. It is somewhat surprising that no disulfide product occurs in the case of the cleavage reaction of **16** (lane 13) and only a little amount in the case of **19** (lane 11). The identity of the two products in the middle of lane 12 and 13 is unclear and whether the upper band represents the SH-octamer also. All mechanisms described in the literature for the cleavage of the P-S-bond involve the formation of either a metaphosphate or a phosphoriodate. Methanol, which was used as a co-solvent for iodine, can attack these reactive intermediates to give a phosphodiester derivative which may account for the product above the 3'-phosphorylated tetramer in lane 12 and 13.²¹ Connolly and Rider failed also to cleave the S-trityl bond of ODNs conjugated at the 5' terminus with trityl-S-(CH₂)_xOH alcohols.²⁰ On the other hand the unsatisfactory cleavage of **16** stands in contradiction to the clean scission of a dithymidine phosphate analog containing a 3'-deoxy-3'-thiothymidine with iodine in aqueous acetone.¹⁰ But if a pyridine containing iodine solution was used thymidine-5'-O-phosphate and an unidentified derivative of 3'-deoxy-3'-thiothymidine was produced. Thus it seems that the iodine promoted cleavage depends very much on the reaction conditions and thus we have not pursued the cleavage with iodine.

SUMMARY AND CONCLUSION

The present work describes a simple way for incorporating a single bridged 5'-phosphorothioate linkage into a specific position of an ODN using automated solid phase phosphoramidite chemistry. For the incorporation we have used a 5'-(S-trityl)-mercapto-5'-deoxythymidine-3'-O-phosphoramidite (**15a,b**). This modified thymidine building block has been utilized in the preparation of the dodecamer **16** which contains a bridged 3'-O-P-S-5' linkage. The P-S bond is stable under the standard synthesis and work up conditions. As expected the P-S bond can be cleaved chemically by the action of 50mM aqueous AgNO₃ or HgCl₂ followed by the reduction of the oxidized oligomer by dithiothreitol. The cleavage reaction proceeds fast and clean and the heavy metal treatment does not harm the natural phosphodiester linkages. The above procedure produces a 3'-phosphate- and a 5'-thiololigomer similar to the cleavage of ODNs containing a bridged 5'-phosphoramidate linkage using 80% CH₃COOH.^{12,13} Furthermore the method is complementary to that described by Cosstick and Vyle for the introduction and cleavage of a 3'-S-P-O-5' linkage.⁹ The incorporation of a P-S linkage into a specific position of an ODN (primer) and the subsequent chemical cleavage could be used for the nicking and manipulation of DNA. After the cleavage reaction the resulting SH-function could be labeled with thiol specific probes or ligated to a polynucleotide or a protein via cleavable disulfide bonds.²⁷ An advantage is that the P-S linkage is achiral. This avoids problems due to generation of diastereoisomers of the modified ODNs.

EXPERIMENTAL

TLC was performed on precoated Merck TLC aluminium sheets with CHCl₃/CH₃OH (90:10, v:v). Melting points are uncorrected.

NMR-Measurements: ¹H-NMR spectra were recorded on a Bruker AM300 WB or WH270 spectrometer. Tetramethylsilane was used as internal reference and the cited chemical shifts are

given in ppm downfield to this standard. ^{31}P -NMR experiments were performed on the AM 300 WB spectrometer. The spectra were recorded at 121.5MHz using broadband proton noise decoupling (reference: 85% H_3PO_4 extern).

Oligodeoxynucleotide synthesis: ODNs were synthesized ($1\mu\text{mol}$ scale) on an Applied Biosystems DNA synthesizer model 380A using the synthesis cycles described in TABLE 2. Deprotection and purification was performed as described previously.²⁸

5'-O-(4-Methylbenzenesulfonyl)-thymidine 13

Thymidine (2.42g, 10mmol) was dissolved in pyridine (50ml) and 4-methylbenzenesulfonyl chloride (3.85g, 20mmol) was added. After three hours at r.t. the reaction was cooled in ice and quenched with water (5ml). After 15 minutes the solution was evaporated and the oily residue was dissolved in ethyl acetate (300ml). The solution was washed with 5% $\text{NaHCO}_{3\text{aq}}$ ($2\times 50\text{ml}$) and sat. brine (100ml), dried with Na_2SO_4 and evaporated in vacuo. The residue was recrystallized from ethanol/ethyl acetate.

Yield: 2.43g (61%), m.p.: 168–170°C, R_f : 0.41, 300MHz- ^1H -NMR ($\text{DMSO}-d_6$): 1.77 (s, 3H, $\text{CH}_3\text{-T}$); 2.12 (m, 2H, 2', 2''-H); 2.41 (s, 3H, $\text{CH}_3\text{-tosyl}$); 3.87 (m, 1H, 4'-H); 4.20 (m, 3H, 5', 5''-H, 3'-H); 5.55 (d, $^3J_{\text{HH}}=4.4\text{Hz}$, 1H, 3'-OH); 6.16 (t, $^3J_{\text{HH}}=6.9\text{Hz}$, 1H, 1'-H); 7.39 (d, 1H, 6-H); 7.48 (d, $^3J_{\text{HH}}=8.3\text{Hz}$, 2H, AA'BB'); 7.80 (d, $^3J_{\text{HH}}=8.3\text{Hz}$, 2H, AA'BB'); 11.34 (s, 1H, NH)

5'-(S-Trityl)-mercapto-5'-deoxythymidine 14

Sodium hydroxide (1.10g, 27.5mmol) was dissolved in water (10ml) and added to a solution of triphenylmethylmercaptan (6.91g, 25mmol) in ethanol (50ml). A solution of 13 (1.98g, 5mmol) in ethanol (40ml) was added and the reaction mixture was stirred under argon at reflux for 8 hours. The suspension was filtered and evaporated in vacuo. The residue was dissolved in ethyl acetate (200ml) and the solution was washed with 5% $\text{NaHCO}_{3\text{aq}}$ ($2\times 50\text{ml}$) followed by saturated brine (100ml), dried with Na_2SO_4 , filtered and evaporated in vacuo. The residual oil was flash chromatographed on silica gel using a step gradient of methanol in methylene chloride (0–5%). Pure fractions were pooled and evaporated to leave a colorless foam.

Yield: 1.40g (56%), R_f : 0.51, 270MHz- ^1H -NMR (CDCl_3): 1.85 (d, $^4J_{\text{HH}}=1.0\text{Hz}$, 3H, CH_3); 2.01 (m, 1H, 2'-H); 2.27 (m, 2H, 2'-H, 3'-OH); 2.48 (dd, $^3J_{\text{HH}}=5.0\text{Hz}$, $^2J_{\text{HH}}=12.8\text{Hz}$, 1H, 5'-H); 2.63 (dd, $^3J_{\text{HH}}=6.1\text{Hz}$, $^2J_{\text{HH}}=12.8\text{Hz}$, 1H, 5''-H); 3.77 (m, 1H, 4'-H); 4.05 (m, 1H, 3'-H); 6.17 (t, $^3J_{\text{HH}}=6.6\text{Hz}$, 1H, 1'-H); 7.20–7.45 (m, 16H, aromat.-H, 6-H); 8.91 (bs, 1H, NH)

5'-(S-trityl)-mercapto-5'-deoxythymidine-3'-O-(2-cyanoethyl, N,N-diisopropylamino)-phosphite 15

To a solution of the protected nucleoside 14 (750mg, 1.5mmol) in dry $\text{CH}_3\text{CN}/\text{CH}_2\text{Cl}_2$ (1:1, v:v, 10ml) under argon was added tetrazole (105mg, 1.5mmol) followed by 2-cyanoethoxy-bis-(N,N-diisopropylamino)-phosphane (750mg, 2.5mmol). After stirring for 1 hour at r.t. the reaction was stopped by adding n-butanol (0.5ml). The solution was diluted with ethyl acetate (150ml), washed with 5% $\text{NaHCO}_{3\text{aq}}$ (20ml) and sat. brine (50ml), shortly dried with Na_2SO_4 (30 minutes) and evaporated in vacuo to an oil. This was dissolved in dry $\text{CH}_2\text{Cl}_2/\text{Et}_2\text{O}$ (1:1, v:v, 10ml) and precipitated into cold n-pentane (200ml).

Yield: 871mg (83%), R_f : 0.72, ^{31}P -NMR (CDCl_3): s at 149.60 and 149.76ppm

Heavy metal cleavage of the backbone modified ODN 16

One A_{260} unit of the oligomer was treated with $10\mu\text{l}$ of a 50mM aqueous solution of AgNO_3 or HgCl_2 at r.t. for 15 minutes. $2.5\mu\text{l}$ of a 220mM solution of dithiothreitol in water was added and the precipitated silver salt was removed by centrifugation. The supernatant was analyzed by PAGE (1mm, 16% PAA, 7M urea, 250V, 6500Vh) after adding of $5\mu\text{l}$ formamide.

Iodine cleavage of the backbone modified ODN 16

One A_{260} unit was treated with $10\mu\text{l}$ of 25mM solution of iodine in $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ (1:1, v:v) for 15 minutes at r.t. $2.5\mu\text{l}$ of a 220mM solution of dithiothreitol was added and after the addition of $5\mu\text{l}$ formamide the mixture was analyzed on a 16% polyacrylamide gel containing 7M urea.

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